

REGULATION OF GENE EXPRESSION USING CHROMATIN
REMODELLING FACTORS

5 The present invention relates to the regulation of gene expression. More particularly, the present invention relates to the control of gene expression of one or more nucleotide sequences of interest in transgenic plants using chromatin remodelling factors.

10 BACKGROUND OF THE INVENTION

Transgenic plants have been an integral component of advances made in agricultural biotechnology. They are necessary tools for the production of plants exhibiting desirable traits (e.g. herbicide and insect resistance, drought and cold
15 tolerance), or producing products of nutritional or pharmaceutical importance. As the applications of transgenic plants become ever more sophisticated, it is becoming increasingly necessary to develop strategies to fine-tune the expression of introduced genes. The ability to tightly regulate the expression of transgenes is important to address many safety, regulatory and practical issues. To this end, it is necessary to
20 develop tools and strategies to regulate the expression of transgenes in a predictable manner.

Several strategies have so far been employed to control plant gene/transgene expression. These include the use of regulated promoters, such as inducible or
25 developmental promoters, whereby the expression of genes of interest is driven by promoters responsive to various regulatory factors (Gatz, 1997). Other strategies involve co-suppression (Eisner et al., 1998) or anti-sense technology (Kohno-Murase et al., 1994), whereby plants are transformed with genes, or fragments thereof, that are homologous to genes either in the sense or antisense orientations. Chimeric RNA-
30 DNA oligonucleotides have also been used to block the expression of target genes in plants (Beetham et al., 1999; Zhu et al., 1999).

Posttranslational modifications of histones in chromatin are important mechanisms in the regulation of gene expression. Protein-protein interactions between histones H3, H4, H2A and H2B form an octomeric core which is wrapped with DNA. N-terminal tails of histones protrude from the octamer and are subject to posttranslational modification involving acetylation and deacetylation of conserved lysine residues. A nucleosome comprises 26 lysine residues that may be subject to acetylation. Acetylation of core histones, including H4 and H3 via histone acetyltransferase (HAT), is correlated with transcriptionally active chromatin of eukaryotic cells. Acetylation is thought to weaken the interactions of histones with DNA and induce alterations in nucleosome structure. These alterations enhance the accessibility of promoters to components of the transcription machinery, and increase transcription. HATs have been identified in yeast, insects, plants and mammals (e.g. Kolle et al. 1998), and are typically components of multiprotein complexes including components of RNA polymerase II complex, TFIID, TFIIC and recruitment factors (e.g. see Lusser et al. 2001 for review).

Histone deacetylation, via histone deacetylase (HD, HDA, HDAC), is thought to lead to a less accessible chromatin conformation, resulting in the repression of transcription (e.g. Pazin and Kadonaga, 1997; Struhl, 1998; Lusser et al., 2001). The role of the yeast histone deacetylase, RPD3, in transcriptional repression was first discovered through a genetic screen for transcriptional repressors in *S. cerevisiae* (Vidal and Gaber, 1991). Since then, a number of yeast and mammalian HDAC genes have been cloned (Rundlett et al., 1996; Emiliani et al., 1998; Hassig et al., 1998; Verdel and Khochbin, 1999). Most eukaryotic histone deacetylases show some sequence homology to yeast RPD3, suggesting that these proteins are all members derived from a single gene family (Khochbin and Wolffe, 1997; Verdel and Khochbin, 1999). In yeast and mammalian cells, the RPD3/HDACs mediate transcriptional repression by interacting with specific DNA-binding proteins or associated corepressors and by recruitment to target promoters (Kadosh and Struhl, 1997; Hassig et al., 1997; Nagy et al., 1997; Gelmetti et al., 1998). Recently, a second family of histone deacetylases, HDA19 and related proteins, were identified in yeast and mammalian cells (Rundlett et al., 1996; Fischle et al., 1999; Verdel and Khochbin, 1999). The deacetylase domain of HDA19-related proteins is homologous to but significantly different from that of RPD3 (Fischle et al., 1999; Verdel and

Khochbin, 1999). These proteins also appear to be functionally different from RPD-like proteins in yeast cells (Rundlett et al., 1996). WO 97/35990 discloses mammalian-derived histone deacetylase (HDx) gene sequences, gene products, and uses for these sequences and products. The down regulation of gene expression in plants using histone deacetylase, fused to a DNA binding domain that targeted the fusion protein to a specific gene, has been demonstrated (Wu et al., 2000a; Wu et al., 2000b).

The present invention embraces the use of fusion proteins comprising a DNA binding domain fused to a recruitment factor, that is capable of recruiting chromatin remodelling proteins such as HDAC and HAT, to specific DNA sites to regulate expression of a gene of interest. Also disclosed is the use of fusion proteins comprising a DNA binding portion fused to histone acetyltransferase (HAT) to regulate transcription of a gene of interest.

It is an object of the invention to overcome disadvantages of the prior art.

The above object is met by the combinations of features of the main claims, the sub-claims disclose further advantageous embodiments of the invention.

SUMMARY OF THE INVENTION

The present invention relates to the regulation of gene expression. More particularly, the present invention relates to the control of gene expression of one or more nucleotide sequences of interest in transgenic plants using chromatin remodelling factors.

According to an aspect of an embodiment of the present invention, there is provided a method to regulate the expression of a gene of interest in a plant comprising:

i) introducing to the plant:

1) a first nucleotide sequence comprising,

a) the gene of interest operatively linked to a first regulatory region,

b) an operator sequence capable of binding a fusion protein, and;

2) a second nucleotide sequence comprising a second regulatory region in operative association with a nucleotide sequence encoding a fusion protein, the fusion protein comprising,

a) a DNA binding protein, or a portion of a DNA binding protein capable of binding the operator sequence, and;

b) a recruitment factor protein, or a portion thereof, capable of binding a chromatin remodelling protein,

ii) growing the plant, wherein expression of the second nucleotide sequence produces the fusion protein and regulates expression of the gene of interest.

The present invention also embraces the methods as defined above, wherein the first and second regulatory regions are either the same or different and are selected from the group consisting of a constitutive promoter, an inducible promoter, a tissue specific promoter, and a developmental promoter.

The present invention also relates to a method of enhancing the expression of a gene of interest or enhancing the transcription of a gene of interest in a plant comprising:

i) introducing to the plant:

1) a first nucleotide sequence comprising,

a) the gene of interest operatively linked to a first regulatory region, and;

b) an operator sequence that interacts with a fusion protein;

2) a second nucleotide sequence comprising a second regulatory region in operative association with a nucleotide sequence encoding a fusion protein comprising,

a) a DNA binding protein, or a portion thereof, capable of binding the operator sequence, and;

b) a histone acetyltransferase (HAT) protein, or portion thereof, capable of increasing histone acetylation;

ii) growing the plant, wherein expression of the second nucleotide sequence produces the fusion protein and increases transcription of the gene of interest.

The present invention pertains to a method of regulating the expression of a gene of interest or enhancing the transcription of a gene of interest in a plant comprising:

i) introducing to the plant:

- 5 1) a first nucleotide sequence comprising,
 - a) the gene of interest operatively linked to a first regulatory region, and;
 - b) an operator sequence that interacts with a fusion protein;
- 10 2) a second nucleotide sequence comprising a second regulatory region in operative association with a nucleotide sequence encoding a fusion protein comprising,
 - a) a DNA binding protein, or a portion thereof, capable of binding the operator sequence, and;
 - b) a chromatin remodelling factor, or portion thereof, capable of
 - 15 increasing histone acetylation;
- ii) growing the plant, wherein expression of the second nucleotide sequence produces the fusion protein and regulates the transcription of the gene of interest.

20 The present invention also embraces the methods as defined above, wherein the first and second regulatory regions are either the same or different and are selected from the group consisting of a constitutive promoter, an inducible promoter, a tissue specific promoter, and a developmental promoter.

25 The first and second nucleotide sequences may be placed within the same or within different vectors, genetic constructs, or nucleic acid molecules. Preferably, the first nucleotide sequence and the second nucleotide sequence are chromosomally integrated into a plant or plant cell. The two nucleotide sequences may be integrated into two different genetic loci of a plant or plant cell, or the two nucleotide sequences may be integrated into a singular genetic locus of a plant or plant cell. However, the

30 second nucleotide sequence may be integrated into the DNA of the plant or it may be present as an extra-chromosomal element, for example, but not wishing to be limiting a plasmid.

Also, according to the present invention there is provided a method for selectively controlling the transcription of a gene of interest, comprising:

- 5 i) producing a first plant comprising a first genetic construct, the first genetic construct comprising a first regulatory region operatively linked to the gene of interest and at least one operator sequence capable of binding a fusion protein;
- ii) producing a second plant comprising a second genetic construct, the second genetic construct comprising a second regulatory region in operative association with a nucleic sequence encoding the fusion protein, the fusion protein comprising,
 - 10 a) a DNA binding protein, or a portion thereof, capable of binding the operator sequence, and;
 - b) a recruitment factor protein, or a portion thereof, capable of binding a chromatin remodelling protein;
- 15 iii) crossing the first plant and the second plant to obtain progeny comprising both the first genetic construct and the second genetic construct, the progeny characterized in that the expression of the fusion protein regulates expression of the gene of interest.

The present invention also embraces the methods as defined above, wherein
20 the first and second regulatory regions are either the same or different and are selected from the group consisting of a constitutive promoter, an inducible promoter, a tissue specific promoter, and a developmental promoter.

The present invention also pertains to the method as just defined, wherein the
25 nucleic acid sequence encoding the fusion protein is optimised for expression in a plant, and that the nucleotide sequence encodes a nuclear localization signal.

Also, according to the present invention there is provided a method for selectively controlling the transcription of a gene of interest, comprising:

- 30 i) producing a first plant comprising a first genetic construct, the first genetic construct comprising a first regulatory region operatively linked to the gene of interest and at least one operator sequence capable of binding a fusion protein;

ii) producing a second plant comprising a second genetic construct, the second genetic construct comprising a second regulatory region in operative association with a nucleic sequence encoding the fusion protein comprising,

a) a DNA binding protein, or a portion thereof, capable of binding the

5 operator sequence, and;

b) a HAT protein, or portion thereof, capable of histone acetylation in plants;

iii) crossing the first plant and the second plant to obtain progeny comprising both the first genetic construct and the second genetic construct and characterized in that the expression of the fusion protein up-regulates the expression of the

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gene of interest.

The present invention also provides the method as just defined, wherein, the nucleic acid sequence encoding the fusion protein is optimised for expression in the

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plant, and that the nucleic acid sequence encodes a nuclear localization signal.

The present invention also embraces the methods as defined above, wherein the first and second regulatory regions are either the same or different and are selected from the group consisting of a constitutive promoter, an inducible promoter, a tissue specific promoter, and a developmental promoter.

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Furthermore, this invention provides a method to regulate expression of an endogenous nucleic acid sequence of interest in a plant comprising:

i) introducing into the plant a nucleotide sequence comprising, a regulatory region, operatively linked with a nucleotide sequence encoding a fusion protein, the fusion protein comprising,

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a) a DNA binding protein, or a portion thereof, capable of binding a segment of a DNA sequence of the endogenous nucleotide sequence of interest;

b) a recruitment factor protein, or a portion thereof, capable of binding a chromatin remodelling protein; and

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ii) growing the plant, wherein expression of the nucleotide sequence produces the fusion protein that regulates expression of the endogenous nucleic acid sequence of interest.

The present invention also includes a method to regulate expression of an endogenous nucleic acid sequence of interest in a plant comprising:

- i) introducing into the plant a nucleotide sequence comprising a regulatory region, operatively linked with a nucleotide sequence encoding a recruitment factor protein, the recruitment factor protein capable of binding an endogenous DNA binding protein, the endogenous DNA binding protein characterized in binding a segment of a DNA sequence of the endogenous nucleotide sequence of interest, and;
- ii) growing the plant, wherein expression of the nucleotide sequence produces the recruitment factor thereby regulating expression of the endogenous nucleic acid sequence of interest.

This summary of the invention does not necessarily describe all necessary features of the invention but that the invention may also reside in a sub-combination of the described features.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features of the invention will become more apparent from the following description in which reference is made to the appended drawings wherein:

- 5 **FIGURE 1** shows the nucleotide and deduced amino acid sequences of wild type ROS and a modified ROS of *Agrobacterium tumefaciens*. **Figure 1(A)** shows the amino acid sequence alignment of known ROS repressors (wild-type ROS, SEQ ID NO:1; ROSR, SEQ ID NO:63; ROSAR, SEQ ID NO: 64; MucR, SEQ ID NO: 65), and a synthetic ROS (SEQ ID NO: 4). The amino acid
- 10 sequence 'PKKKRKV' (SEQ ID NO: 6) at the carboxy end of synthetic ROS is one of several nuclear localization signals. **Figure 1(B)** shows the nucleotide sequence of a synthetic ROS (SEQ ID NO:2) that had been optimised for plant codon usage containing a nuclear localization signal peptide (in italics). Optional restriction sites at the 5' end of the sequence are
- 15 underlined. **Figure 1(C)** shows the consensus nucleotide (SEQ ID NO:3) and predicted amino acid (SEQ ID NO:4) sequence, of a composite ROS sequence comprising all possible nucleotide sequences that encode wild type ROS repressor, and the wild type ROS amino acid sequence. The amino acid
- 20 sequence 'PKKKRKV' (SEQ ID NO:6) at the carboxy end represents a nuclear localization signal. Amino acids in bold identify the zinc finger motif. Nucleotide codes are as follows: N= A or C or T or G; R= A or G; Y= C or T; M= A or C; K= T or G; S= C or G; W= A or T; H= A or T or C; B= T or C or G; D= A or T or G; V= A or C or G. **Figure 1(D)** shows the nucleotide sequence of the operator sequences of the virC/virD (SEQ ID NO:27) and ipt
- 25 (SEQ ID NO:8) genes. **Figure 1(E)** shows a consensus operator sequence (SEQ ID NO:5) derived from the virC/virD (SEQ ID NOs:66-67) and ipt (SEQ ID NOs:68-69) operator sequences. This sequence comprises 10 amino acids, however, only the first 9 amino acids are required for binding ROS.
- 30 **FIGURE 2-4** shows in a diagrammatic form several variations of regulating gene expression using the methods of the present invention.

FIGURE 5 shows schematic representations of nucleotide constructs that place the expression of a gene of interest under the control a regulatory region, in this

case a CaMV35S regulatory region, modified to contain a ROS operator site. **Figure 5(A)** shows the nucleotide construct p74-315 in which a CaMV35S regulatory region, modified to contain a ROS operator site downstream of the TATA box, is operatively linked to a gene of interest (β -glucuronidase; GUS). **Figure 5(B)** shows the nucleotide construct p74-316 in which a CaMV35S regulatory region is modified to contain a ROS operator site upstream of the TATA box is operatively linked to the protein encoding region of GUS. **Figure 5(C)** shows the nucleotide construct p74-309 in which a CaMV35S regulatory region modified to contain ROS operator sites upstream and downstream of the TATA box is transcriptionally fused (i.e. operatively linked) to the protein encoding region of GUS. **Figure 5(D)** shows construct p74-118 comprising a 35S regulatory region with three ROS operator sites downstream from the TATA box. The 35S regulatory region is operatively linked to the gene of interest (GUS).

FIGURE 6 shows a schematic representation of a nucleotide construct that places the expression of a gene of interest gene under the control of a regulatory region, in this case, the tms2 regulatory region that has been modified to contain ROS operator sites. **Figure 6(A)** shows the nucleotide construct p76-507 in which a tms2 regulatory region is operatively linked to a gene of interest (in this case encoding β -glucuronidase, GUS). **Figure 6(B)** shows the nucleotide construct p76-508 in which a tms2 regulatory region modified to contain two tandemly repeated ROS operator sites downstream of the TATA box is transcriptionally fused (i.e. operatively linked) to the protein coding region of GUS.

FIGURE 7 shows a schematic representation of a nucleotide construct that places the expression of a gene of interest under the control of a regulatory region, in this case actin 2 regulatory region, that has been modified to contain ROS operator sites. **Figure 7(A)** shows the nucleotide construct p75-101 in which an actin2 regulatory region is operatively linked to a gene of interest (the β -glucuronidase (GUS) reporter gene). **Figure 7(B)** shows the nucleotide construct p74-501 in which an actin2 regulatory region modified to contain two tandemly repeated ROS operator sites upstream of the TATA box is transcriptionally fused (operatively linked) to the a gene of interest (GUS).

FIGURE 8 shows Southern analysis of transgenic *Arabidopsis* plants. **Figure 8(A)** shows Southern analysis of a plant comprising a first genetic construct, p74-309 (35S-operator sequence-GUS; see Figure 5(C) for map). DNA was digested with *Cla*I or *Xho*I and the blot was probed with the ORF of the GUS gene. **Figure 8(B)** shows Southern analysis of a plant comprising a second genetic construct p75-101 (see Figure 7A). *Hind*III digests were probed with NPTII.

FIGURE 9 shows expression of a gene of interest in plants. Upper panel shows expression of GUS under the control of 35S (pBI121; 35S:GUS). Middle panel shows GUS expression under the control of actin2 comprising ROS operator sequences (p74-501; see Figure 7(B) for construct). Lower panel shows the lack of GUS activity in a non-transformed control.

FIGURE 10 shows alignments of bnKCP1 and sequence comparison of kinase inducible domains (KIDs) in bnKCP1 and CREB family members. **Figure 10(A)** shows alignment of the deduced amino acid sequences of bnKCP1 (SEQ ID NO:71), atKCP (SEQ ID NO:72), atKCL1 (SEQ ID NO:73) and atKCL2 (SEQ ID NO:74) proteins. Serine (S)-rich residues and the conserved region (GKSKS domain) among the four sequences are single underlined and double underlined, respectively. The putative nuclear localization signal (NLS) and the phosphorylation site of protein kinase A are indicated by asterisks and diamonds, respectively. **Figure 10(B)** shows alignment of the amino acid sequences of bnKCP1 (SEQ ID NO:75), hydra CREB (hyCREB) (SEQ ID NO:77), canfa CREM (cCREM) (SEQ ID NO:80), and mammalian ATF-1 (SEQ ID NO:76), CREB (SEQ ID NO:78) and CREM (SEQ ID NO:79). Diamonds indicate the conserved phosphorylation site of protein kinase A. **Figure 10(C)** shows a phylogenetic tree of the KIDs sequences using the NTI Vector program.

FIGURE 11 shows structural features of bnKCP1. **Figure 11(A)** shows schematic representation of entire bnKCP1 protein. Numbers above or under the boxes refer to positions of amino acid residues. S-rich (34-58), GKSKS (88-143) and

KID (161-215) domains or motifs are shown in dotted boxes, the nuclear localization signal (NLS) in black box, and the three acidic motifs (I, II, III) in gray boxes.

Figure 11(B) shows secondary structure features and hydrophilicity of bnKCP1 analyzed using DNASTar Protean program.

FIGURE 12 shows Southern blot analysis of Brassica genomic DNA. Total genomic DNA (10 µg/lane) from *Brassica napus* cv Westar was digested with restriction enzymes *Eco*RI (EI), *Xba*I (X), *Hind*III (H), *Pst*I (P), *Eco*RV (EV) and *Kpn*I (K). The entire ORF of *bnKCP1* was used as a probe.

FIGURE 13 shows *in vitro* interaction of wild type and mutant bnKCP1 proteins with the GST-HDA19 and GST-Gcn5 fusion proteins. Figure 13(A) shows a schematic representation of the bnKCP1 and its deletion mutants obtained by deletion of C-terminal regions of bnKCP1. Figure 13(B) shows binding activities of bnKCP1 and its mutants with GST-HDA19, GST-Gcn5 and GST alone (negative control), respectively, as indicated. The wild type bnKCP1, mutants bnKCP1¹⁻¹⁶⁰ and bnKCP1¹⁻⁸⁰, luciferase (as positive control) and negative control (no template) were produced using *in vitro* transcription/translation reactions. The translation products were incubated with GST fusion proteins or GST and their binding activities were examined as described in Example 4. Figure 13(C) shows activation of lacZ reporter gene by bnKCP1 and its deletion mutants, ΔbnKCP1¹⁻¹⁶⁰ and ΔbnKCP1¹⁻⁸⁰, in yeast cells. MaV203 yeast cells carrying plasmid pDBLeu-HDA19 and the reporter gene were transfected with the plasmid pPC86-bnKCP1, pPC86-bnKCP1¹⁻¹⁶⁰, pPC86-bnKCP1¹⁻⁸⁰ or pPC86 vector only. Yeast strains A and B were used as negative and positive controls, respectively. The β-galactosidase activity was assayed using chlorophenol red-β-D-galactopyranoside (CPRG) and was expressed as a percentage of activity conveyed by bnKCP1.

FIGURE 14 shows the effect of S¹⁸⁸ on the interaction between bnKCP1 and GST-HDA19 fusion protein. A glycine residue (G¹⁸⁸) was introduced by site-directed mutagenesis to replace S¹⁸⁸. The binding activities of wild-type bnKCP1 and the mutant ΔbnKCP1G¹⁸⁸ with GST-HDA19 or GST alone (negative control) were examined with GST pulldown affinity assay as

described in Example 4. Figure 14A shows the introduction of G188 into the KID of bnKCP1. Figure 14B shows in vitro protein interaction of bnKCP1 and the mutant Δ bnKCP1G¹⁸⁸ with GST-HDA19 or GST alone.

5 **FIGURE 15** shows expression patterns of *bnKCP1* mRNA in different tissues. Total RNA (20 μ g/lane) was isolated from leaves with petioles, flowers, roots, stems and immature siliques.

FIGURE 16 shows expression of *bnKCP1* gene in response to low temperature,
10 LaCl_3 and inomycin treatments. Total RNA (20 μ g/lane) was isolated from leaf blades of four-leaf stage *Brassica napus* cv Westar seedlings after exposure to different stress conditions and analyzed by northern blotting using the *bnKCP1* ORF as probe. **Figure 16(A)** shows *bnKCP1* transcript accumulation in leaves and stems of seedlings exposed to cold (4°C). **Figure**
15 **16(B)** shows expression pattern of *bnKCP1* gene after treatment with LaCl_3 and inomycin.

FIGURE 17 shows transactivation of the *lacZ* gene by bnKCP1 in yeast. The *lacZ* gene was driven by a promoter containing GAL4 DNA binding sites and integrated into the genome of yeast MaV203. **Figure 17(A)** is a schematic representation of the bnKCP1 and its deletion mutants. **Figure 17(B)** Yeast cells carrying the reporter gene were transfected with the effector plasmids pDBLeu-bnKCP1, pDBLeu-bnKCP1¹⁻¹⁶⁰, pDBLeu-bnKCP1¹⁻⁸⁰, and pDBLeu-bnKCP1⁸¹⁻²¹⁵ or the pDBLeu vector only. Yeast strains A and B (GibcoL
20 BRL, Life Technologies) were used as negative and positive controls, respectively. The β -galactosidase activity was assayed using CPRG (chlorophenol red- β -D-galactopyranoside) and was expressed as a percentage of activity conveyed by the positive control (strain C). Bars indicate the standard error of three replicates.

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FIGURE 18 shows the nuclear localization of GUS-bnKCP1 protein in onion cells. **Figure 18(A)** is a schematic diagram of the GUS-bnKCP1 fusion construct containing the CaMV 35S promoter. The *bnKCP1* was fused in-frame to the GUS reporter gene. **Figure 18(B)** shows transient expression of GUS-

bnKCP1 fusion protein (top) and GUS alone (bottom) in onion cells. Onion tissues were simultaneously analysed using histochemical GUS assay (left) and nucleus-specific staining with DAPI (right) as described in Example 4.

5 **FIGURE 19** shows a diagrammatic representation of a strategy for preparing fusions between a recruitment factor involved in chromatin remodelling and a DNA binding protein. In the non-limiting example shown in this figure, the recruitment factor is KID (see Example 4), and the DNA binding protein is a zinc finger.

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FIGURE 20 shows alignment of the deduced products of *BnSCL1* (SEQ ID NO:81), *AtSCL15* (accession number Z99708) (SEQ ID NO:82) and *LsSCL* (accession number AF273333) (SEQ ID NO:83). Identical and conserved amino acids in the three proteins are shown as white letters on a black background and black letter on a gray background, respectively. Amino acids with weak similarity are indicated as white letters on a gray background. Amino acids with no similarity are shown as black letters on a white background. The putative nuclear localization signals and LXXLL motif are indicated by asterisks and dots, respectively. The VHIIID motif, two leucine heptad regions (LHRI and LHRII), PFYRE and SAW motif are underlined as indicated.

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FIGURE 21 shows a phylogenetic tree of the GRAS family sequences made by the NTI Vector program in *Brassica napus*, *Arabidopsis thaliana*, *Hordeum vulgare*, *Zea mays*, *Lycopersicon esculentum*, *Pisum sativum* and *Oryza sativa*. The *BnSCL1* is underlined.

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FIGURE 22 shows DNA gel blot analysis of *BnSCL1* gene. Total genomic DNA (10 µg/lane) from *Brassica napus* was digested with restriction enzymes *EcoRI* (EI), *XbaI* (X), *HindIII* (H), *PstI* (P), *EcoRV* (EV) and *KpnI* (K), and hybridized with the entire ORF of *BnSCL1* under high stringency conditions.

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FIGURE 23 shows *in vitro* interaction of wild type and mutant *BnSCL1* proteins with the GST-HDA19 fusion protein. **Figure 23(A)** is a schematic representation of the *BnSCL1* and its deletion mutants obtained by the deletion of its C-terminal regions.

Figure 23(B) shows the binding activities of BnSCL1 and its mutants with GST-HDA19.

The wild type BnSCL1, mutants Δ BnSCL1¹⁻³⁵⁸, Δ BnSCL1¹⁻²⁶¹, Δ BnSCL1¹⁻²¹⁷ and Δ BnSCL1¹⁻¹⁴⁵, luciferase (positive control) and negative control (no template) were produced using *in vitro* transcription/translation reactions. The translation products were incubated with GST fusion proteins or GST alone (data not shown) and their binding activities were examined as described in Example 5. Arrow point to band representing the *in vitro* translated Δ BnSCL1¹⁻¹⁴⁵ protein that did not bind to the recombinant protein.

FIGURE 24 shows *in vivo* interaction of wild type and mutant BnSCL1 proteins. Figure

24(A) is a schematic representation of the BnSCL1 and its deletion mutants. Figure 24(B) shows the activation of *lacZ* reporter gene by BnSCL1 and its deletion mutants in yeast cells. MaV203 yeast cells carrying plasmid pDBLeu-HDA19 and the *lacZ* reporter gene were transfected with the plasmid pPC86-BnSCL1, pPC86-BnSCL1¹⁻³⁵⁸, pPC86-BnSCL1¹⁻²⁶¹, pPC86-BnSCL1¹⁻²¹⁷, pPC86-BnSCL1¹⁻¹⁴⁵, pPC86-BnSCL1¹⁴⁶⁻³⁵⁸, pPC86-BnSCL1²¹⁸⁻⁴³⁸ or pPC86 vector only. The negative control yeast strain A, and the positive controls yeast strains B and C (GIBCOL BRL, Life Technologies) were also used. The β -Galactosidase activity was assayed using CPRG (chlorophenol red- β -D-galactopyranoside) and was expressed as a percentage of activity conveyed by yeast strain C. Bars indicate the standard error of three replicates.

FIGURE 25 shows transactivation of the *lacZ* gene by BnSCL1 protein in yeast. Figure

25(A) is a schematic representation of the BnSCL1 and its deletion mutants. Figure 25(B) shows the activation of *lacZ* reporter gene by BnSCL1 and its deletion mutants in yeast cells. The *lacZ* reporter gene was driven by a promoter containing GAL4 DNA binding sites and integrated into the genome of yeast MaV203 cell. Yeast cells carrying the reporter gene were transfected with the effector plasmids pDBLeu-BnSCL1, pDBLeu-BnSCL1¹⁻³⁵⁸, pDBLeu-BnSCL1¹⁻²⁶¹, pDBLeu-BnSCL1¹⁻²¹⁷, pDBLeu-BnSCL1¹⁻¹⁴⁵, pDBLeu-BnSCL1¹⁴⁶⁻³⁵⁸, pDBLeu-BnSCL1²¹⁸⁻⁴³⁸ or pDBLeu vector only. Yeast strains A, B, C and D (GIBCOL BRL, Life Technologies) were used as controls as described in Example 5. The β -Galactosidase activity was assayed using CPRG and was expressed as a percentage

of activity conveyed by the wild type BnSCL1 protein. Bars indicate the standard error of three replicates.

FIGURE 26 shows expression patterns of *BnSCL1* mRNA in different tissues. **Figure 26(A)** is a RNA gel blot analysis of total RNA (20 µg/lane) isolated from leaves, flowers, roots, stems and siliques, electrophoresed through a 1.2% agarose gel containing formaldehyde and probed with the ORF of *BnSCL1* as described in Example 5. EtBr stained total RNA is shown to indicate even loading. **Figure 26(B)** is a quantitative one-step RT-PCR analysis of total RNA extracted from leaves, flowers, roots, stems, siliques and shoots. Quantitative RT-PCR products were electrophoresed through a 1% agarose gel and hybridized with ³²P-labelled 5'-end fragment (435 bp) of *BnSCL1* ORF. A 960 bp fragment of the *Brassica napus actin* gene co-amplified with *BnSCL1* was used as an internal standard as described in Example 5.

FIGURE 27 shows expression of *BnSCL1* gene in four-leaf stage *Brassica napus* seedlings in the presence or absence of 2,4-D. Total RNA was isolated from the fourth leaves after the indicated period of the first foliar application of 1 mM 2,4-D and subjected to quantitative one-step RT-PCR. The RT-PCR products were analyzed by Southern blotting using the *BnSCL1* ORF as probe (left) and the blotting results were shown graphically relative to the level of internal standard *Actin* (arbitrary value of 100)(right).

FIGURE 28 shows kinetics of *BnSCL1* mRNA accumulation in response to auxin in the presence and absence of histone deacetylase inhibitor sodium butyrate. Nine-day-old light-grown seedlings were treated with 10 mM sodium butyrate for 24 h followed by exogenous 2,4-D application at variable concentrations as indicated. Quantitative one-step RT-PCR was used to analyze total RNA extracted from shoots (**Figure 28A**) and roots (**Figure 28B**) (see legend to Figure 27 Expression of *BnSCL1* in response to 2,4-D was also analyzed using quantitative RT-PCR of total RNA isolated from shoots and roots of 10 dpg seedlings in the presence of 50 µM NPA, an auxin transport inhibitor, for 24 h before the exogenous application of 2,4-D (**Figure 28C**).

FIGURE 29 shows in a diagrammatic form several constructs that may be used to regulate gene expression as described in Example 6.

DESCRIPTION OF PREFERRED EMBODIMENT

The present invention relates to the regulation of gene expression. More particularly, the present invention relates to the control of gene expression of one or more nucleotide sequences of interest in transgenic plants using chromatin remodelling factors.

The following description is of a preferred embodiment by way of example only and without limitation to the combination of features necessary for carrying the invention into effect.

Gene regulation can be used in applications such as metabolic engineering to produce plants that accumulate large amounts of certain intermediate compounds. Regulation of gene expression can also be used for control of transgenes across generations, or production of F1 hybrid plants with seed characteristics that would be undesirable in the parental line, for example but not limited to, hyper-high oil, reduced fiber content, low glucosinolate levels, reduced levels of phytotoxins, and the like. In the latter examples, low glucosinolate levels, or other phytotoxins, may be desired in seeds while higher concentrations of these compounds may be required elsewhere, for example in the case of glucosinolates, within cotyledons, due to their role in plant defence. Another non-limiting example for the controlled regulation of a gene of interest during plant development is seed specific down regulation of sinapine biosynthesis, as for example in seeds of *Brassica napus*. In many instances, transgene expression needs to be regulated only in certain plant organs/tissues or at certain stages of development. The methods as described herein may also be used to control the expression of a gene of interest that encodes a protein used to for plant selection purposes. For example, which is to be considered non-limiting, a gene of interest may encode a protein that is capable of metabolizing a compound from a non-toxic form to a toxic form thereby selectively removing plants that express the gene of interest.

The present invention provides a method to regulate the expression of a gene of interest by transforming a plant with one or more constructs comprising:

- 1) a first nucleotide sequence comprising,

a) a nucleic acid sequence of interest operatively linked to a regulatory region,

b) an operator sequence capable of binding a fusion protein, and;

2) a second nucleotide sequence comprising a regulatory region in operative association with a nucleotide sequence encoding a fusion protein, the fusion protein comprising,

a) a DNA binding protein, or a portion of a DNA binding protein capable of binding the operator sequence, and;

b) a recruitment factor protein, or a portion of a recruitment factor protein capable of binding a chromatin remodelling protein,

wherein binding of the fusion protein to the operator sequence of the first nucleotide sequence regulates expression of the nucleic acid sequence of interest from the first nucleotide sequence. The operator sequence of the first nucleotide sequence may be positioned upstream of the ORF of the nucleic acid sequence of interest.

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These first and second nucleotide sequences may be placed within the same or within different vectors, genetic constructs, or nucleic acid molecules. Preferably, the first nucleotide sequence and the second nucleotide sequence are chromosomally integrated into a plant or plant cell. The two nucleotide sequences may be integrated into two different genetic loci of a plant or plant cell, or the two nucleotide sequences may be integrated into a singular genetic locus of a plant or plant cell. However, the second nucleotide sequence may be integrated into the DNA of the plant or it may be present as an extra-chromosomal element, for example, but not wishing to be limiting a plasmid.

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By "operatively linked" it is meant that the particular sequences interact either directly or indirectly to carry out their intended function, such as mediation or modulation of gene expression. The interaction of operatively linked sequences may, for example, be mediated by proteins that in turn interact with the sequences. A transcriptional regulatory region and a sequence of interest are "operably linked" when the sequences are functionally connected so as to permit transcription of the sequence of interest to be mediated or modulated by the transcriptional regulatory region.

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By the term "regulate the expression" it is meant reducing or increasing the level of mRNA, protein, or both mRNA and protein, encoded by a gene or nucleotide sequence of interest in the presence of the fusion protein encoded by the second nucleotide sequence, relative to the level of mRNA, protein or both mRNA and protein encoded by the nucleic acid sequence of interest in the absence of the fusion protein encoded by the second nucleotide sequence.

By the term "fusion protein" it is meant a protein comprising two or more amino acid portions which are not normally found together within the same protein in nature and that are encoded by a single gene. Fusion proteins may be prepared by standard techniques in molecular biology known to those skilled in the art (see for example Figure 17). In the context of the present invention, at least one of the amino acid portions is capable of binding an operator sequence as defined herein.

By the term "binding" it is meant reversible or non-reversible association of two components, for example the operator sequence and the DNA binding domain of a protein, including a fusion protein, or the recruitment factor protein and chromatin remodelling protein as described herein. Preferably, the two components have a tendency to remain associated, but are capable of dissociation under appropriate conditions. Conditions may include, but are not limited to the addition of a third component, chemical, etc which enhances dissociation of the bound components.

By the term "recruitment factor" it is meant a protein or peptide sequence capable of interacting with, or binding a chromatin remodelling protein. Preferably, the recruitment factor and the chromatin remodelling protein interact or bind in a manner such that the activity of the chromatin remodelling protein is retained. However, by binding the recruitment factor, the activity of the chromatin remodelling protein may be modified in some manner. Non-limiting examples of recruitment factors include KID, for example bnKCP1, or fragments thereof (Example 4), BnSCL1, or fragments thereof (Example 5), ADA, SAGA, STAGA, PCAF, TFIID, and TFIIC (Lusser, 2001, Table 1, which is incorporated herein by reference). A recruitment factor may be modified to include a DNA binding region, for example as outlined in Figure 17, [or a native recruitment factor may be utilized to target proteins that interact with genes in their native context]. An example, which is not to be

considered limiting in any manner, bnKCP1, or active fragments thereof (see Example 4) can target transcription factors that are known to bind DNA. Examples of such transcription factors include ERF (Hart et al., 1993), SEBF (Boyle and Brisson, 2001), or CBF (Stockinger et al., 1997). In this manner by over expressing *bnKCP1*,
5 regulation of the expression of a gene that is dependant on ERF, CBF or SEBF activity may be regulated. Another non-limiting example of a recruitment factor is BnSCL1, or active fragments thereof (see Example 5). An example, which is not to be considered limiting, of a protein that interacts with bnKCP1 and BnSCL1 is the chromatin remodelling protein HDAC, for example HDA19.

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By the term "chromatin remodelling protein" it is meant a protein that is capable of altering the structure of chromatin. Preferably the chromatin remodelling protein is histone acetyl transferase (HAT) or histone deacetylase (referred to either as HD, HDA, or HDAC). Any HAT protein, HDAC protein, or any derivative of any
15 HAT protein or HDAC protein may be used in the method of the present invention provided that the HAT protein, HDAC protein or derivative thereof exhibits the respective histone acetylase, or histone deacetylase activity in plants.

By the term "HD binding domain" or "histone deacetylase binding domain", it
20 is meant a sequence of amino acid residues which interacts with a histone deacetylase enzyme through protein-protein interactions. Such protein-protein interactions can be monitored in several ways, for example, which is not to be considered limiting, by yeast two-hybrid experiments. Non-limiting examples of proteins comprising a HD binding domain include bnKCP1 and BnSCL1.

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By the term "DNA binding protein or portion of a DNA binding protein" it is meant a protein or amino acid sequence capable of binding to a specific operator sequence. By "operator sequence" it is meant a sequence of DNA that is capable of binding to the DNA binding protein or portion of the DNA binding protein. Examples
30 of a DNA binding proteins capable of binding specific operator sequences include, but are not limited to, the ROS repressor, TET repressor, Sin3, VP16, GAL4, Lex A, UMe6, ERF, SEBF and CBF. Any DNA binding protein or portion of any DNA binding protein may be employed in the method of the present invention provided that the protein or portion thereof is capable of binding to an operator sequence. As an

example, but not to be considered limiting in any manner, the ROS repressor may be employed in the method of the present invention. By ROS repressor it is meant any ROS repressor, analog or derivative thereof as known within the art which is capable of binding to an operator sequence. These include ROS repressors as described herein, as well as other microbial ROS repressors, for example but not limited to ROSAR (*Agrobacterium radiobacter*; Brightwell et al., 1995) (SEQ ID NO:64), MucR (*Rhizobium meliloti*; Keller M et al., 1995) (SEQ ID NO:65), and ROSR (*Rhizobium elti*; Bittinger et al., 1997; also see Cooley et al., 1991; Chou et al., 1998; Archdeacon J et al., 2000; D'Souza-Ault M. R., 1993; all of which are incorporated herein by reference) (SEQ ID NO:63). The DNA sequence of ROS, or any other DNA binding protein, may be modified to optimize expression within a plant. Examples of ROS repressors that may be used as described herein are provided in Figures 1(A) to (C) and (SEQ ID NOs: 1-4).

The DNA binding protein, or portion thereof that exhibits DNA binding activity may be fused to a recruitment factor or chromatin remodelling protein as described herein. Examples of such fusion proteins can be prepared, using methods known in the art, for example but not limited to the method outlined in Figure 17. Figure 17 discloses a strategy for creating fusion between the zinc finger domain of the ROS repressor and the KID domain of bnKCP1. This involves amplification of regions encoding the zinc finger domain of the ROS repressor and the KID domain using the following primers:

zinc finger: The forward primer (zf-F) contains a restriction enzyme site at the 5' end and the reverse primer (zf-R) contains 15 nucleotides from the 5' end of the KID region.;

KID domain: The forward primer (KID-F) contains 15 nucleotides from the 3' region of the zinc finger domain, and the reverse primer (KID-R) contains a restriction enzyme site at the 3' end.

The amplified zinc finger and KID fragments are combined and used as a template for a new round of PCR amplification where only the forward primer (zf-F) of the zinc finger and the reverse primer (KID-R) of the KID domain are used. The two separate templates are amplified to create one single in frame fusion fragment encoding the zinc finger and KID domains, and containing restriction sites at each end. This product is then cloned into a plant expression vector.

However, it is to be understood that fusion of a recruitment factor with a DNA binding protein may not be required in order to regulate expression of a nucleic acid sequence of interest. Recruitment factors are known to bind chromatin remodelling proteins and factors that directly or indirectly bind DNA. For example, bnKCP1 (Example 4) exhibits the property of binding ERF.

Depending upon the chromatin remodelling protein selected, gene expression may be up-regulated or down-regulated. For example, which is not to be considered limiting in any manner, the binding of a fusion protein containing a recruitment factor capable of recruiting HAT to a gene, may result in up-regulation of expression of a nucleic acid sequence of interest, while a fusion protein that recruits HDAC will result in the down-regulation of the expression of a nucleic acid sequence of interest. However, it is within the scope of the present invention that modification to the rate of up-regulation and down-regulation of gene expression may occur depending upon the location of the operator sequence that binds the fusion protein.

The operator sequence is preferably located in proximity to the nucleic acid sequence of interest, either upstream of or downstream of the nucleic acid sequence of interest (see for example Figure 5A-D). Alternatively, the operator sequence may be within the non-coding region of the nucleic acid sequence of interest, for example, but not wishing to be limiting, within an intron of the gene. If it is desired to have the expression of a nucleic acid sequence of interest reduced or repressed, the operator sequence may be located within a nucleotide region that interferes with binding of transcription factors required for transcription of the nucleic acid sequence of interest, for example, interfering with the binding of the RNA polymerase to the nucleic acid sequence of interest, or reducing the rate of migration of the polymerase along a nucleotide sequence, or both.

An operator sequence may consist of a minimal sequence required for binding of a DNA binding protein or fragment thereof, or it may comprise an inverted repeat or palindromic sequences of a specified length. For example, but not wishing to be limiting, the ROS operator sequence may comprise 9 or more nucleotide base pairs (see Figures 1 (D) and (E)) that exhibits the property of binding a DNA binding

domain of a ROS repressor. A consensus sequence of a 10 amino acid region including the 9 amino acid DNA binding site sequence is WATDHWKMAR (SEQ ID NO: 5; Figure 1 (E)). The last amino acid, "R", of the consensus sequence is not required for ROS binding (data not presented). Examples of operator sequences, which are not to be considered limiting in any manner, also include, as is the case with the ROS operator sequence from the *virC* or *virD* gene promoters, a ROS operator made up of two 11bp inverted repeats separated by TTTA:

TATATTTCAATTTTATTGTAATATA (SEQ ID NO: 7); or

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the operator sequence of the *IPT* gene:

TATAATTAAAATATTAAGTCTCGCATT (SEQ ID NO: 8).

15 However, it is to be understood that analogs or variants of SEQ ID NO's:7, 8 and 5 may also be used providing they exhibit the property of binding a DNA binding domain, preferably a DNA binding domain of the ROS repressor. For example, but not to be considered limiting in any manner, in the promoter of the divergent *virC/virD* genes of *Agrobacterium tumefaciens*, ROS binds to a 9 bp inverted repeat sequence in an orientation-independent manner (Chou et al., 1998). The ROS operator sequence in the *ipt* promoter also consists of a similar sequence to that in the *virC/virD* except that it does not form an inverted repeat (Chou et al., 1998). Only the first 9 bp are homologous to ROS box in *virC/virD* indicating that the second 9 bp sequence may not be a requisite for ROS binding. Accordingly, the use of ROS operator sequences or variants thereof that retain the ability to interact with ROS, as operator sequences to selectively control the expression of genes or nucleotide sequences of interest, is within the scope of the present invention.

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Other operator sequences include sequences known to bind transcription factors, for example but not limited to:

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TAAGAGCCGCC (SEQ ID NO:9), which is known to bind ERF (in ethylene responsive genes; Hart et al., 1993);

GACTGTCAC (SEQ ID NO:10), which is known to bind to SEBF (in pathogenesis responsive genes; Boyle and Brisson, 2001);

TACCGACAT (SEQ ID NO:11) and TGGCCGAC (SEQ ID NO:12), which are known to bind CBF (in low temperature responsive genes; Stockinger et al., 1997).

5 The transcription factors ERF, SEBF and CBF are example of factors that can be targeted by the recruitment factor bnKCP1.

By "regulatory region" or "regulatory element" it is meant a portion of nucleic acid typically, but not always, upstream of the protein coding region of a gene, which may be comprised of either DNA or RNA, or both DNA and RNA. When a
10 regulatory region is active and in operative association with a nucleic acid sequence of interest, this may result in expression of the nucleic acid sequence of interest. A regulatory element may be capable of mediating organ specificity, or controlling developmental or temporal gene activation. A "regulatory region" includes promoter elements, core promoter elements exhibiting a basal promoter activity, elements that
15 are inducible in response to an external stimulus, elements that mediate promoter activity such as negative regulatory elements or transcriptional enhancers. "Regulatory region", as used herein, also includes elements that are active following transcription, for example, regulatory elements that modulate gene expression such as translational and transcriptional enhancers, translational and transcriptional
20 repressors, upstream activating sequences, and mRNA instability determinants. Several of these latter elements may be located proximal to the coding region.

In the context of this disclosure, the term "regulatory element" or "regulatory region" typically refers to a sequence of DNA, usually, but not always, upstream (5')
25 to the coding sequence of a structural gene, which controls the expression of the coding region by providing the recognition for RNA polymerase and/or other factors required for transcription to start at a particular site. However, it is to be understood that other nucleotide sequences, located within introns, or 3' of the sequence may also contribute to the regulation of expression of a coding region of interest. An example
30 of a regulatory element that provides for the recognition for RNA polymerase or other transcriptional factors to ensure initiation at a particular site is a promoter element. Most, but not all, eukaryotic promoter elements contain a TATA box, a conserved nucleic acid sequence comprised of adenosine and thymidine nucleotide base pairs usually situated approximately 25 base pairs upstream of a transcriptional start site. A

promoter element comprises a basal promoter element, responsible for the initiation of transcription, as well as other regulatory elements (as listed above) that modify gene expression.

5 There are several types of regulatory regions, including those that are developmentally regulated, inducible or constitutive. A regulatory region that is developmentally regulated, or controls the differential expression of a gene under its control, is activated within certain organs or tissues of an organ at specific times during the development of that organ or tissue. However, some regulatory regions
10 that are developmentally regulated may preferentially be active within certain organs or tissues at specific developmental stages, they may also be active in a developmentally regulated manner, or at a basal level in other organs or tissues within the plant as well.

15 An inducible regulatory region is one that is capable of directly or indirectly activating transcription of one or more DNA sequences or genes in response to an inducer. In the absence of an inducer the DNA sequences or genes will not be transcribed. Typically the protein factor, which binds specifically to an inducible regulatory region to activate transcription, may be present in an inactive form which is
20 then directly or indirectly converted to the active form by the inducer. However, the protein factor may also be absent. The inducer can be a chemical agent such as a protein, metabolite, growth regulator, herbicide or phenolic compound or a physiological stress imposed directly by heat, cold, salt, or toxic elements or indirectly through the action of a pathogen or disease agent such as a virus. A plant
25 cell containing an inducible regulatory region may be exposed to an inducer by externally applying the inducer to the cell or plant such as by spraying, watering, heating or similar methods. Inducible regulatory elements may be derived from either plant or non-plant genes (e.g. Gatz, C. and Lenk, I.R.P.,1998; which is incorporated by reference). Examples, of potential inducible promoters include, but not limited to,
30 tetracycline-inducible promoter (Gatz, C.,1997; which is incorporated by reference), steroid inducible promoter (Aoyama, T. and Chua, N.H.,1997; which is incorporated by reference) and ethanol-inducible promoter (Salter, M.G., et al, 1998; Caddick, M.X. et al,1998; which are incorporated by reference) cytokinin inducible IB6 and CKII genes (Brandstatter, I. and Kieber, J.J.,1998; Kakimoto, T., 1996; which are

incorporated by reference) and the auxin inducible element, DR5 (Ulmasov, T., et al., 1997; which is incorporated by reference).

5 A constitutive regulatory region directs the expression of a gene throughout the various parts of a plant and continuously throughout plant development. Examples of known constitutive regulatory elements include promoters associated with the CaMV 35S transcript. (Odell et al., 1985), the rice actin 1 (Zhang et al, 1991), actin 2 (An *et al.*, 1996), or tms 2 (U.S. 5,428,147, which is incorporated herein by reference), and triosephosphate isomerase 1 (Xu et. al., 1994) genes, the
10 maize ubiquitin 1 gene (Cornejo et al, 1993), the *Arabidopsis* ubiquitin 1 and 6 genes (Holtorf et al, 1995), and the tobacco translational initiation factor 4A gene (Mandel et al, 1995). The term "constitutive" as used herein does not necessarily indicate that a gene under control of the constitutive regulatory region is expressed at the same level in all cell types, but that the gene is expressed in a wide range of cell types even
15 though variation in abundance is often observed.

The regulatory regions of the first and second nucleotide sequences denoted above, may be the same or different. For example, which is not to be considered limiting in any manner, the regulatory elements of the first and second genetic
20 constructs may both be constitutive. In an aspect of an embodiment, the first and second nucleotide sequences may be maintained in the same plant. In an alternate embodiment the first and second nucleotide sequences are maintained in separate plants, a first and a second plant, respectively. The first nucleotide sequence encoding a nucleic acid sequence of interest is expressed within the first plant. In the second
25 embodiment, the second plant expresses the second nucleic acid sequence encoding the fusion protein capable of regulating the expression of the nucleic acid sequence of interest within the first plant. Crossing of the first and second plants produces a progeny that expresses the fusion protein which regulates the expression of the nucleic acid sequence of interest. In this manner the expression of nucleic acid
30 sequence of interest that is required to maintain parent stocks may be retained within a parent plant but not expressed in a progeny plant. Such a cross may produce sterile offspring.

Alternatively, which is not to be considered limiting in any manner, the second regulatory element may be active before, during, or after the first regulatory element is active. Similarly, the first regulatory element may be active before, during, or after the second regulatory element is active. Other examples, which are not to be considered limiting, include the second regulatory element being an inducible regulatory element that is activated by an external stimulus so that regulation of gene expression may be controlled through the addition of an inducer. The second regulatory element may also be active during a specific developmental stage preceding, during, or following that of the activity of the first regulatory element. In this way the expression of the nucleic acid sequence of interest may be repressed or activated as desired within a plant.

By "nucleic acid sequence of interest", "nucleotide sequence of interest" or "coding region of interest" it is meant any gene or nucleotide sequence that is to be expressed within a host organism. Such a nucleotide sequence of interest may include, but is not limited to, a gene whose product has an effect on plant growth or yield, for example a plant growth regulator such as an auxin or cytokinin and their analogues, or a nucleotide sequence of interest may comprise a herbicide or a pesticide resistance gene, which are well known within the art. A nucleic acid sequence of interest or a coding region of interest, may encode an enzyme involved in the synthesis of, or in the regulation of the synthesis of, a product of interest, for example, but not limited to a protein, or an oil product. A nucleotide sequence of interest or a coding region of interest, may encode an industrial enzyme, protein supplement, nutraceutical, or a value-added product for feed, food, or both feed and food use. Examples of such proteins include, but are not limited to proteases, oxidases, phytases, chitinases, invertases, lipases, cellulases, xylanases, enzymes involved in oil biosynthesis, etc.

A nucleotide sequence of interest or a coding region of interest, may also encode a pharmaceutically active protein, for example growth factors, growth regulators, antibodies, antigens, their derivatives useful for immunization or vaccination and the like. Such proteins include, but are not limited to, interleukins, insulin, G-CSF, GM-CSF, hPG-CSF, M-CSF or combinations thereof, interferons, for example, interferon- α , interferon- β , interferon- γ , blood clotting factors, for example, Factor VIII, Factor IX, or tPA or combinations thereof. If the nucleic acid sequence

of interest or a coding region of interest, encodes a product that is directly or indirectly toxic to the plant, then by using the method of the present invention, such toxicity may be reduced throughout the plant by selectively expressing the nucleic acid sequence of interest within a desired tissue or at a desired stage of plant development.

A nucleotide sequence of interest or a coding region of interest, may also include a gene that encodes a protein involved in regulation of transcription, for example DNA-binding proteins that act as enhancers or basal transcription factors. Moreover, a nucleotide sequence of interest may be comprised of a partial sequence or a chimeric sequence of any of the above genes, in a sense or antisense orientation.

It is also contemplated that a nucleic acid sequence of interest or a coding region of interest, may be involved in the expression of a gene expression cascade, for example but not limited to a developmental cascade. In this embodiment, the nucleic acid sequence of interest is preferably associated with a gene that is involved at an early stage within the gene cascade, for example homeotic genes. Expression of a nucleic acid sequence of interest, for example a repressor of homeotic gene expression, represses the expression of a homeotic gene. Expression of the fusion protein that represses gene expression within the same plant, either via crossing, induction, temporal or developmental expression of the regulatory region, as described herein, de-represses the expression of the homeotic gene thereby initiating a gene cascade. Conversely, using the methods described herein, expression of an introduced (i.e. transgenic) homeotic gene may be activated in a selective manner, so that it is expressed outside of its normal developmental or temporal expression pattern, thereby initiating a cascade of developmental events. This may be achieved by targeting a chromatin remodelling protein to a desired homeotic gene as described herein.

Homeotic genes are well known to one of skill in the art, and include but are not limited to, transcription factor proteins and associated regulatory regions, for example controlling sequences that bind AP2 domain containing transcription factors, for example but not limited to, APETALA2 (a regulator of meristem identity, floral organ specification, seedcoat development and floral homeotic gene expression;

Jofuku *et al.*, 1994), CCAAT box-binding transcription factors (e.g. *LEC1*; WO 98/37184; Lotan, T. *et al.*, 1998), or the controlling factor associated with *PICKLE*, a gene that produces a thickened, primary root meristem (Ogas, J. *et al.*, 1997).

5 A nucleic acid sequence of interest or a coding region of interest, may also be involved in the control of transgenes across generations, or production of F1 hybrid plants with seed characteristics that would be undesirable in the parental line or progeny, for example but not limited to, oil seeds characterized as having reduced levels of sinapine biosynthesis within the oil-free meal. In this case, a nucleic acid
10 sequence of interest may be any enzyme involved in the synthesis of one or more intermediates in sinapine biosynthesis. An example, which is to be considered non-limiting, is caffeic o-methyltransferase (Acc# AAG51676), which is involved in ferulic acid biosynthesis. Other examples of genes of interest include genes that encode proteins involved in fiber, or glucosinolate, biosynthesis, or a protein involved
15 in the biosynthesis of a phytotoxin. Phytotoxins may also be used for plant selection purposes. In this non-limiting example, a nucleic acid sequence of interest may encode a protein that is capable of metabolizing a compound from a non-toxic form to a toxic form thereby selectively removing plants that express the nucleic acid sequence of interest. The phytotoxic compound may be synthesized from endogenous
20 precursors that are metabolized by the nucleic acid sequence of interest into a toxic form, for example plant growth regulators, or the phytotoxic compound may be synthesized from an exogenously applied compound that is only metabolized into a toxic compound in the presence of the nucleic acid sequence of interest. For example, which is not to be considered limiting, the nucleic acid sequence of interest may
25 comprise indole acetamide hydrolase (IAH), that converts exogenously applied indole acetamide (IAM) or naphthaline acetamide (NAM), to indole acetic acid (IAA), or naphthaline acetic acid (NAA), respectively. Over-synthesis of IAA or NAA is toxic to a plant, however, in the absence of IAH, the applied IAM or NAM is non-toxic. Similarly, the nucleic acid sequence of interest may encode a protein involved in
30 herbicide resistance, for example, but not limited to, phosphinothricin acetyl transferase, wherein, in the absence of the gene encoding the transferase, application of phosphinothricin, the toxic compound (herbicide) results in plant death. Other nucleic acid sequence of interest that encode lethal or conditionally lethal products may be found in WO 00/37660 (which is incorporated herein by reference).

The nucleic acid sequence of interest, the nucleotide sequence of interest or a coding region of interest, may be expressed in suitable eukaryotic hosts which are transformed by the nucleotide sequences, or nucleic acid molecules, or genetic constructs, or vectors of the present invention. Examples of suitable hosts include, but are not limited to, insect hosts, mammalian hosts, yeasts and plants. Suitable plant hosts include, but are not limited to agricultural crops including canola, *Brassica* spp., maize, tobacco, alfalfa, rice, soybean, wheat, barley, sunflower, and cotton.

The one or more chimeric genetic constructs of the present invention can further comprise a 3' untranslated region. A 3' untranslated region refers to that portion of a gene comprising a DNA segment that contains a polyadenylation signal and any other regulatory signals capable of effecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by effecting the addition of polyadenylic acid tracks to the 3' end of the mRNA precursor. Polyadenylation signals are commonly recognized by the presence of homology to the canonical form 5'-AATAAA-3' although variations are not uncommon. One or more of the chimeric genetic constructs of the present invention can also include further enhancers, either translation or transcription enhancers, as may be required. These enhancer regions are well known to persons skilled in the art, and can include the ATG initiation codon and adjacent sequences. The initiation codon must be in phase with the reading frame of the coding sequence to ensure translation of the entire sequence.

Examples of suitable 3' regions are the 3' transcribed non-translated regions containing a polyadenylation signal of *Agrobacterium* tumor inducing (Ti) plasmid genes, such as the nopaline synthase (Nos gene) and plant genes such as the soybean storage protein genes and the small subunit of the ribulose-1, 5-bisphosphate carboxylase (ssRUBISCO) gene.

To aid in identification of transformed plant cells, the constructs of this invention may be further manipulated to include selectable markers. Useful selectable markers in plants include enzymes which provide for resistance to chemicals such as an antibiotic for example, gentamycin, hygromycin, kanamycin, or herbicides such as

phosphinothrycin, glyphosate, chlorosulfuron, and the like. Similarly, enzymes providing for production of a compound identifiable by colour change such as GUS (β -glucuronidase), or luminescence, such as luciferase or GFP, are useful.

5 Also considered part of this invention are transgenic eukaryotes, for example but not limited to plants containing the chimeric gene construct of the present invention. However, it is to be understood that the chimeric gene constructs of the present invention may also be combined with nucleic acid sequence of interest for expression within a range of eukaryotic hosts.

10

In instances where the eukaryotic host is a plant, methods of regenerating whole plants from plant cells are also known in the art. In general, transformed plant cells are cultured in an appropriate medium, which may contain selective agents such as antibiotics, where selectable markers are used to facilitate identification of transformed plant cells. Once callus forms, shoot formation can be encouraged by employing the appropriate plant hormones in accordance with known methods and the shoots transferred to rooting medium for regeneration of plants. The plants may then be used to establish repetitive generations, either from seeds or using vegetative propagation techniques. Transgenic plants can also be generated without using tissue cultures (for example, Clough and Bent, 1998).

20

The constructs of the present invention can be introduced into plant cells using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, micro-injection, electroporation, etc. For reviews of such techniques see for example Weissbach and Weissbach, 1988; Geierson and Corey, 1988; and Miki and Iyer, 1997; Clough and Bent, 1998). The present invention further includes a suitable vector comprising the chimeric gene construct.

25

The DNA binding protein which is employed in the method of the present invention may be naturally produced in an organism other than a plant. For example, but not wishing to be considered limiting, a ROS repressor is encoded by a nucleotide sequence of bacterial origin and, as such the nucleotide sequence may be optimised, for example, by changing its codons to favour plant codon usage, by attaching a nucleotide sequence encoding a nuclear localisation signal (NLS), for example but not

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limited to SV40 localization signal (see Robbins *et al.*, 1991; Rizzo, P., *et al.*, 1991; which are incorporated herein by reference) in order to improve the efficiency of ROS transport to the plant nucleus to facilitate the interaction with its respective operator, or both optimizing plant codon usage. Addition of an NLS to a fusion protein comprising a binding domain, for example the ROS repressor binding domain, and a recruitment factor, may also ensure targeting of the fusion product to the nuclear compartment. Similar optimization may be performed for other DNA binding proteins of non-plant source, however, such optimization may not always be required. Other possible nuclear localization signals that may be fused to a DNA binding protein include but are not limited to those listed in Table 1:

Table 1: nuclear localization signals

	Nuclear Protein	Organism	NLS	Ref
15	AGAMOUS	A	RienttnrqvtfcKRR (SEQ ID NO:13)	1
	TGA-1A	T	RRlaqnreaaRKsRIRKK (SEQ ID NO:14)	2
	TGA-1B	T	KKRaRlvrnresaqlsRqRKK (SEQ ID NO:15)	2
	O2 NLS B	M	RKRKsresnaRRsRyRK (SEQ ID NO:16)	3
	NIa	V	KKnqkhklkm-32aa-KRK (SEQ ID NO:17)	4
20	Nucleoplasmin	X	KRpaaatkkagqaKKKKI (SEQ ID NO:18)	5
	NO38	X	KRIapdsaskvpRKKtR (SEQ ID NO:19)	5
	N1/N2	X	KRKteesplKdKdaKK (SEQ ID NO:20)	5
	Glucocorticoid			
	receptor	M,R	RkclqagmnleaRKtKK (SEQ ID NO:21)	5
25	a receptor	H	RKclqagmnleaRKtKK (SEQ ID NO:22)	5
	β receptor	H	RKclqagmnleaRKtKK (SEQ ID NO:23)	5
	Progesterone receptor	C,H,Ra	RKccqagmvlggRKfKK (SEQ ID NO:24)	5
	Androgen receptor	H	RKcyeagmtlgaRKIKK (SEQ ID NO:25)	5
	p53	C	RRcfervrcacpgRdRK (SEQ ID NO:26)	5

⁺A, *Arabidopsis*; X, *Xenopus*; M, mouse; R, rat; Ra, rabbit; H, human; C, chicken; T, tobacco; M, maize; V, potyvirus.

References:

1. Yanovsky *et al.*, 1990

2. van der Krol and Chua, 1991
3. Varagona *et al.*, 1992
4. Carrington *et al.*, 1991
5. Robbins *et al.*, 1991

5

Incorporation of a nuclear localization signal into the fusion protein of the present invention may facilitate migration of the fusion protein, into the nucleus. Without wishing to be bound by theory, reduced levels of fusion proteins elsewhere
10 within the cell may be important when the DNA binding portion of the fusion protein may bind analogue operator sequences within other organelles, for example within the mitochondrion or chloroplast. Furthermore, the use of a nuclear localization signal may permit the use of a less active promoter or regulatory region to drive the expression of the fusion protein while ensuring that the concentration of the expressed
15 protein remains at a desired level within the nucleus, and that the concentration of the protein is reduced elsewhere in the cell.

Referring now to Figures 2A-C, there is shown aspects of an embodiment of the method of the present invention. Shown in Figure 2A are two constructs which
20 have been introduced within a plant cell. The constructs comprise:

- 1) a first nucleotide sequence (10) comprising,
 - a) a nucleic acid sequence of interest (20) operatively linked to a first regulatory region (30);
 - b) an operator sequence (40) capable of binding a fusion protein (85,
25 Figure 2B), and;
- 2) a second nucleotide sequence (60) comprising a second regulatory region (70) in operative association with a nucleotide sequence (80) encoding a fusion protein (85).

The fusion protein (Figure 2B; 85) encoded by nucleotide sequence (80) comprises
30 a) a DNA binding protein (100), or a portion of a DNA binding protein capable of binding the operator sequence (40, Figure 2A), and;
b) a recruitment factor protein (110), or a portion of a recruitment factor protein capable of binding a chromatin remodelling protein (120), for example but not limited to histone deacetylase, HDAC.

In the example shown in Figure 2 A-C, the operator sequence (40) is shown as being upstream from the regulatory region (30), however, the operator sequence may also be positioned downstream from the regulatory region (40), for example between the regulatory region (40) and the nucleic acid sequence of interest (20; see for example the constructs in Figure 5A-D), within the coding region of the nucleic acid sequence of interest (20), or downstream of the nucleic acid sequence of interest (20).

Referring now to Figure 2C, but without wishing to be bound by theory, transcription and translation of nucleotide sequence (60; Figure 2A) produces fusion protein (80; Figure 2B) which is capable of binding operator sequence (40; Figure 2A) and for example, histone deacetylase (120). Dual binding of histone deacetylase (120) to fusion protein (85) and fusion protein (85) to operator sequence (40) facilitates enzymatic deacetylation of histones (via bound histone deacetylase) in proximity of the nucleic acid sequence of interest (20) thereby causing repression of the nucleic acid sequence of interest (20).

The first (10) and second (60) nucleotide sequences may be placed within the same or within different vectors, genetic constructs, or nucleic acid molecules. Preferably, the first nucleotide sequence and the second nucleotide sequence are chromosomally integrated into a plant or plant cell. The two nucleotide sequences may be integrated into two different genetic loci of a plant or plant cell, or the two nucleotide sequences may be integrated into a singular genetic locus of a plant or plant cell. However, the second nucleotide sequence may be integrated into the DNA of the plant or it may be present as an extra-chromosomal element, for example, but not wishing to be limiting a plasmid. Furthermore, the first and second regulatory regions may be the same or different, and maybe active in a constitutive, temporal, developmental or inducible manner.

Referring now to Figures 3A-C, there is shown aspects of an alternate embodiment of the method of the present invention. Shown in Figure 3A are two constructs which have been introduced into a plant cell. The constructs comprise:

- 1) a first nucleotide sequence (10) comprising,
 - a) a nucleic acid sequence of interest (20) operatively linked to a regulatory region (30),

b) an operator sequence (40) capable of binding a fusion protein (85, Figure 3B), and;

2) a second nucleotide sequence (60) comprising a regulatory region (70) in operative association with a nucleotide sequence (80) encoding a fusion protein (85).

The fusion protein (85) encoded by nucleotide sequence (80) comprises,

a) a DNA binding protein (100), or a portion of a DNA binding protein capable of binding the operator sequence (40), and;

b) a recruitment factor protein (110), or a portion of a recruitment factor protein capable of binding a chromatin remodelling protein, for example but not limited, to free histone acetyltransferase (HAT) (120).

In the example shown in Figure 3 A-C, the operator sequence (40) is shown as being upstream from the regulatory region (30), however, the operator sequence may also be positioned downstream from the regulatory region (40), for example between the regulatory region (40) and the nucleic acid sequence of interest (20; see for example the constructs in Figure 5A-D), within the coding region of the nucleic acid sequence of interest (20), or downstream of the nucleic acid sequence of interest (20).

Referring now to Figure 3C, but without wishing to be bound by theory, transcription and translation of nucleotide sequence (80; Figure 3A) produces fusion protein (85; Figure 3B) which is capable of binding operator sequence (40; Figure 3A) and free histone acetyltransferase (120). Dual binding of histone acetyltransferase (120) to fusion protein (85) and fusion protein (85) to operator sequence (40) facilitates enzymatic acetylation of histones (via bound histone acetyltransferase) in proximity of the nucleic acid sequence of interest (20) thereby causing an increase in the transcription of the nucleic acid sequence of interest (20).

The present invention also relates to a method of enhancing the expression of a nucleic acid sequence of interest or enhancing the transcription of one or more selected nucleotide sequences by transforming a plant with one or more constructs comprising:

1) a first nucleotide sequence comprising,

a) a nucleic acid sequence of interest operatively linked to a regulatory region, and;

b) an operator sequence that interacts with a fusion protein;

2) a second nucleotide sequence comprising a regulatory region in operative association with a nucleotide sequence encoding a fusion protein comprising,

a) a DNA binding protein, or a portion of a DNA binding protein capable of binding the operator sequence, and;

b) a histone acetyltransferase (HAT) protein, or portion of a histone acetyltransferase protein which is capable of increasing histone acetylation;

and wherein binding of the fusion protein to the operator sequence increases histone acetylation in the proximity of the nucleic acid sequence of interest within the first nucleotide sequence thereby increasing the transcription of the nucleic acid sequence of interest.

These first and second nucleotide sequences may be placed within the same or within different vectors, genetic constructs, or nucleic acid molecules. Preferably, the first nucleotide sequence and the second nucleotide sequence are chromosomally integrated into a plant or plant cell. The two nucleotide sequences may be integrated into two different genetic loci of a plant or plant cell, or the two nucleotide sequences may be integrated into a singular genetic locus of a plant or plant cell. However, the second nucleotide sequence may be integrated into the DNA of the plant or it may be present as an extra-chromosomal element, for example, but not wishing to be limiting a plasmid, or transiently expressed, for example when using viral vectors, biolistics for transformation.

Preferably, the operator sequence is located in a nucleotide region that does not sterically hinder binding of transcription factors to the regulatory region, binding of the RNA polymerase to the nucleic acid sequence of interest, or migration of the polymerase along the DNA of the first nucleotide sequence, nucleic acid sequence of interest or both.

Referring now to Figures 4A-C, there is shown aspects of an embodiment of the method of the present invention. Shown in Figure 4A are two constructs which have been introduced within a plant cell. The constructs comprise:

1) a first nucleotide sequence (10) comprising,

- a) a nucleic acid sequence of interest (20) operatively linked to a regulatory region (30),
 - b) an operator sequence (40) capable of binding a fusion protein (85), and;
- 5 2) a second nucleotide sequence (60) comprising a regulatory region (70) in operative association with a nucleotide sequence (80) encoding a fusion protein (85).

The fusion protein (85) encoded by nucleotide sequence (80) comprises

- 10 a) a DNA binding protein (100), or a portion of a DNA binding protein capable of binding the operator sequence (40), and;
- b) a histone acetyltransferase protein (130), or a portion of a histone acetyltransferase protein.

15 Referring now to Figure 4C, but without wishing to be bound by theory, transcription and translation of nucleotide sequence (80; Figure 4A) produces fusion protein (85; Figure 4B) which comprises an active HAT protein (130), or portion thereof. Binding of the fusion protein (85) to the operator sequence facilitates enzymatic acetylation of histones in proximity to the nucleic acid sequence of interest

20 (20) thereby enhancing the expression of a nucleic acid sequence of interest.

In the example shown in Figure 4 A-C, the operator sequence (40) is shown as being upstream from the regulatory region (30), however, the operator sequence may also be positioned downstream from the regulatory region (40), for example between

25 the regulatory region (40) and the nucleic acid sequence of interest (20; see for example the constructs in Figure 5A-D), within the coding region of the nucleic acid sequence of interest (20), or downstream of the nucleic acid sequence of interest (20).

Also contemplated by the present invention is the control of gene expression

30 accomplished through combinations of activator, effector and gene of interest constructs as outlined in Figures 29 A and B (see Example 6). With reference to Figure 29A, the expression of a gene of interest (reporter) is regulated using three constructs:

a reporter construct (or gene of interest construct),

an activator construct and
an effector construct.

The gene of interest construct includes a gene of interest, for example but not limited to a reporter gene (e.g. the *lacZ* gene), in operative association with a regulatory
5 element and an operator sequence.

The activator construct comprises a nucleic acid sequence encoding a recruitment factor protein, or a portion thereof, capable of binding a chromatin remodelling protein, fused with a nucleotide sequence encoding a DNA binding
10 protein, or a fragment thereof. The recruitment factor protein may be, for example but not limited to BnSCL1, bnKCP1 or an active fragment thereof; the DNA binding protein could be, for example but not limited to VP16 or GAL4 DNA Binding domain. In this case the activator construct produces a VP16-bNSCL1 fusion protein.

15 The effector plasmid includes a nucleic acid sequence encoding a chromatin remodelling factor, for example but not limited to HDA19, operatively associated with a regulatory element and a nucleic acid sequence encoding a nuclear localisation signal. The constructs are expressed in eukaryotes, for example plant, animal or yeast.

20 When the activator construct is co-expressed with the gene of interest (reporter) construct, the DNA binding sequence binds the operator sequence of the gene of interest construct. This results in modification in the expression of the gene of interest due to interaction of the activator protein within the transcriptional
25 machinery. In this example, the activator protein is fused to a recruitment factor protein, and the VP16-BnSCL1 fusion protein binds the Tet operator sequence of the gene activator construct resulting in increased expression of the gene of interest.

30 Co-expression of the effector construct, inconjunction with the gene of interest and activator constructs, results in synthesis of a chromatin remodelling factor, in this case HAD19, which associates with the recruitment factor protein, BnSCL1. Association of HDAC with the construct expressing the gene of interest, reduces expression of the gene of interest.

In a second aspect, the expression of a gene of interest is regulated using two constructs: a gene of interest (reporter)+activator and an effector construct as shown in Figure 29B. Expression of the reporter+activator construct results in an increased expression of the gene of interest due to binding of the activator portion of the construct to the operator sequence of the gene of interest construct. This association may be inhibited in the presence of tetracycline. As in the case outlined with reference to Figure 29A, above, co-expression of the effector construct results in reduced expression of the gene of interest due to association of HDAC to the activator-recruitment factor fusion protein (VP16-BnSCL1 fusion)

10

The present invention also provides for a method to regulate expression of a nucleic acid sequence of interest, wherein the nucleic acid sequence of interest comprises an endogenous sequence. In this embodiment, a nucleotide sequence comprising a regulatory region in operative association with a nucleotide sequence encoding a recruitment factor, or a portion thereof, that is known to interact with a factor that binds the nucleic acid sequence of interest, is expressed in the host. The recruitment factor protein, or a portion thereof is capable of binding a chromatin remodelling protein, for example but not limited, HDAC or HAT, and the recruitment factors also interacts with endogenous factors that bind the nucleotide sequence of interest (e.g. transcription factors). In this manner, expression of the recruitment factor in a temporal, tissue specific, or induced manner will result in the expression of the recruitment factor that binds the chromatin remodelling factor and transcription factor resulting in modulation of expression of the nucleic acid sequence of interest. A non-limiting example of this embodiment includes the expression of bnKCP1 and its interaction with HDAC and transcription factors ERF, SEBF or CBF.

25

Therefore, the present invention provides a method to regulate expression of an endogenous nucleic acid sequence of interest in a plant comprising:

i) introducing into the plant a nucleotide sequence comprising, a regulatory region, operatively linked with a nucleotide sequence encoding a recruitment factor protein, the recruitment factor protein capable of binding an endogenous DNA binding protein, the endogenous DNA binding protein characterized in binding a segment of a DNA sequence of the endogenous nucleotide sequence of interest, and;

30

ii) growing the plant, wherein expression of the nucleotide sequence produces the recruitment factor thereby regulating expression of the endogenous nucleic acid sequence of interest.

5 An alternate embodiment of the present invention includes a method to regulate expression of an endogenous nucleic acid sequence of interest. In this example, a DNA binding protein, or a portion thereof, known to interact with the DNA of an endogenous nucleic acid sequence of interest is fused to a chromatin remodelling factor. Expression of the fusion protein permits the recruitment factor
10 portion of the fusion protein to interact or bind with a chromatin remodelling, for example but not limited to HDAC or HAT, and the DNA binding portion of the fusion protein binds the nucleotide sequence of interest. In this manner, expression of the fusion protein in a temporal, tissue specific, or induced manner will result in the expression of a recruitment factor that binds a chromatin remodelling factor and the
15 DNA of a nucleic acid sequence of interest, resulting in modulation of expression of the endogenous nucleic acid sequence of interest. Examples of DNA binding proteins, or portions thereof, that bind endogenous nucleic acid sequences of interest, which are not to be considered limiting, include ERF, SEBF or CBF. A non-limiting example of a recruitment factor is bnKCP1 or BnSCL1.

20 Therefore, the present invention also provides a method to regulate expression of an endogenous nucleic acid sequence of interest in a plant comprising:

i) introducing into the plant a nucleotide sequence comprising, a regulatory region, operatively linked with a nucleotide sequence encoding a fusion protein, the fusion
25 protein comprising,

- a) a DNA binding protein, or a portion thereof, capable of binding a segment of a DNA sequence of the endogenous nucleotide sequence of interest, and;
- b) a recruitment factor protein, or a portion thereof, capable of binding a chromatin remodelling protein; and

30 ii) growing the plant, wherein expression of the nucleotide sequence produces the fusion protein that regulates expression of the endogenous nucleic acid sequence of interest.

Also contemplated by the present invention is a method of increasing cold tolerance in a plant. The method comprises providing a plant having a nucleotide sequence of interest operatively linked to a first regulatory region; the nucleotide sequence of interest encodes bnKCP1, or a fragment thereof. The plant is maintained under conditions where bnKCP1 is expressed. In this manner, the plant expressing bnKCP1 is preconditioned for cold adaptation and exhibits increased cold tolerance.

By the term cold in the context of cold tolerance, it is meant a temperature in the range of about -10°C to about 10°C . An example of cold temperature, without wishing to be limiting, is a temperature in the range of about -8°C to about 8°C ; a further example is a temperature of about -10 to about -1°C .

Sequences of the present invention are listed in Table 2.

15

Table 2

SEQ ID NO:1	aa seq of wild-type ROS (<i>A. tumefaciens</i>)	Fig 1A (WT-ROS)
SEQ ID NO:2	Nucl seq synthetic ROS optimized for plant, with NLS	Fig 1B
SEQ ID NO:3	Consensus nucl seq of composite ROS	Fig 1C
SEQ ID NO:4	aa seq of synthetic ROS	Fig 1A, 1C
SEQ ID NO:5	ROS binding sequence	Fig 1E
SEQ ID NO:6	aa seq of NLS (PKKKRKV)	
SEQ ID NO:7	ROS operator sequence	
SEQ ID NO:8	IPT gene operator sequence	
SEQ ID NO:9	Operator sequence binding to ERF	
SEQ ID NO:10	Operator sequence binding to SEBF	
SEQ ID NO:11	Operator sequence binding to CBF	
SEQ ID NO:12	Operator sequence binding to CBF	
SEQ ID NO:13	NLS of AGAMOUS protein	Table 1, page 30
SEQ ID NO:14	NLS of TGA-1A protein	Table 1, page 30
SEQ ID NO:15	NLS of TGA-1B protein	Table 1, page 30
SEQ ID NO:16	NLS of O2 NLS B protein	Table 1, page 30
SEQ ID NO:17	NLS of N1a protein	Table 1, page 30
SEQ ID NO:18	NLS of nucleoplasmin protein	Table 1, page 30
SEQ ID NO:19	NLS of NO38 protein	Table 1, page 30
SEQ ID NO:20	NLS of N1/N2 protein	Table 1, page 30
SEQ ID NO:21	NLS of Glucocorticoid receptor	Table 1, page 30
SEQ ID NO:22	NLS of Glucocorticoid a receptor	Table 1, page 30
SEQ ID NO:23	NLS of Glucocorticoid b receptor	Table 1, page 30
SEQ ID NO:24	NLS of Progesterone receptor	Table 1, page 30
SEQ ID NO:25	NLS of Androgen receptor	Table 1, page 30

SEQ ID NO:26	NLS of p53 protein	Table 1, page 30
SEQ ID NO:27	VirC/VirD operator seq	Fig 1D
SEQ ID NO:28	ROS-OPDS, p74-315	
SEQ ID NO:29	ROS-OPDA, p74-315	
SEQ ID NO:30	ROS-OPUS, p74-316	
SEQ ID NO:31	ROS-OPUA, p74-316	
SEQ ID NO:32	ROS-OPPS, p74-309	
SEQ ID NO:33	ROS-OPPA, p74-309	
SEQ ID NO:34	ROS-OP1, p74-508	
SEQ ID NO:35	ROS-OP2, p74-508	
SEQ ID NO:36	tms2 promoter sense primer, p74-508	
SEQ ID NO:37	tms2 promoter anti-sense primer, p74-508	
SEQ ID NO:38	Actin2 promoter sense primer, p74-501	
SEQ ID NO:39	Actin2 promoter anti-sense primer, p74-501	
SEQ ID NO:40	p74-315 seq from EcoRV to ATG of GUS	
SEQ ID NO:41	p74-316 seq from EcoRV to ATG of GUS	
SEQ ID NO:42	p74-309 seq from EcoRV to ATG of GUS	
SEQ ID NO:43	p74-118 seq from EcoRV to ATG of GUS	
SEQ ID NO:44	Forward primer for HDA19 <i>A. thaliana</i> , pDBLeu-HDA19	
SEQ ID NO:45	Reverse primer for HDA19 <i>A. thaliana</i> , pDBLeu-HDA19	
SEQ ID NO:46	Forward primer for Gcn5 Arabidopsis, GST-Gcn5	
SEQ ID NO:47	Reverse primer for Gcn5 Arabidopsis, GST-Gcn5	
SEQ ID NO:48	Reverse primer for HDA19, GST-HDA19	
SEQ ID NO:49	Forward-primer for bnKCP1, 1-80, 1-160 (generation of mutants)	
SEQ ID NO:50	Reverse primer for bnKCP1 1-160 (generation of mutants)	
SEQ ID NO:51	Reverse primer for bnKCP1 1-80 (generation of mutants)	
SEQ ID NO:52	Reverse primer for bnKCP1 (generation of mutants)	
SEQ ID NO:53	Forward primer for bnKCP1, 1-80 and 1-160 (in vivo assay and transactivation assay)	
SEQ ID NO:54	Reverse primer for bnKCP1 (in vivo assay and transactivation assay) and 81-215 (transactivation assay)	
SEQ ID NO:55	Reverse primer for bnKCP1 1-160 (in vivo assay and transactivation assay)	
SEQ ID NO:56	Reverse primer for bnKCP1 1-80 (in vivo assay and transactivation assay)	
SEQ ID NO:57	Forward primer for bnKCP1G188	
SEQ ID NO:58	Reverse primer for bnKCP1G188	
SEQ ID NO:59	Forward primer for bnKCP1 81-215 (transactivation assay)	
SEQ ID NO:60	Forward primer for entire coding region of bnKCP1	
SEQ ID NO:61	Reverse primer for entire coding region of bnKCP1	
SEQ ID NO:62	pat7 NLS (PLNKKRR)	
SEQ ID NO:63	aa seq of ROSR (ROS repressor)	Fig 1A
SEQ ID NO:64	aa seq of ROSAR (ROS repressor)	Fig 1A
SEQ ID NO:65	aa seq of MucR (ROS repressor)	Fig 1A
SEQ ID NO:66	VirC/VirD DNA binding site seq (1)	Fig 1D
SEQ ID NO:67	VirC/VirD DNA binding site seq (2)	Fig 1D
SEQ ID NO:68	ipt DNA binding site seq (1)	Fig 1D

SEQ ID NO:69	ipt DNA binding site seq (2)	Fig 1D
SEQ ID NO:70	Consensus DNA binding site seq	Fig 1D
SEQ ID NO:71	bnKCP aa seq	Fig 10A
SEQ ID NO:72	atKCP aa seq	Fig 10A
SEQ ID NO:73	atKCL1 aa seq	Fig 10A
SEQ ID NO:74	atKCL2 aa seq	Fig 10A
SEQ ID NO:75	bnKCP aa seq	Fig 10B
SEQ ID NO:76	ATF-1 aa seq	Fig 10B
SEQ ID NO:77	hyCREB aa seq	Fig 10B
SEQ ID NO:78	CREB aa seq	Fig 10B
SEQ ID NO:79	CREM aa seq	Fig 10B
SEQ ID NO:80	cCREM aa seq	Fig 10B
SEQ ID NO:81	aa seq of BnSCL1	Fig 20
SEQ ID NO:82	aa seq of atSCL15	Fig 20
SEQ ID NO:83	aa seq of lsSCR	Fig 20
SEQ ID NO:84	BnSCL1 sense primer	
SEQ ID NO:85	BnSCL1 anti-sense primer	
SEQ ID NO:86	BnIAA1 sense primer	
SEQ ID NO:87	BnIAA1 anti-sense primer	
SEQ ID NO:88	BnIAA12 sense primer	
SEQ ID NO:89	BnIAA12 anti-sense primer	
SEQ ID NO:90	Forward primer for <i>BnSCL1</i> , <i>BnSCL1</i> ¹⁻³⁵⁸ , <i>BnSCL1</i> ¹⁻²⁶¹ , <i>BnSCL1</i> ¹⁻²¹⁷ and <i>BnSCL1</i> ¹⁻¹⁴⁵ for pET-28b vector	
SEQ ID NO:91	Reverse primer for <i>BnSCL1</i> for pET-28b vector	
SEQ ID NO:92	Reverse primer for <i>BnSCL1</i> ¹⁻³⁵⁸ for pET-28b vector	
SEQ ID NO:93	Reverse primer for <i>BnSCL1</i> ¹⁻²⁶¹ for pET-28b vector	
SEQ ID NO:94	Reverse primer for <i>BnSCL1</i> ¹⁻²¹⁷ for pET-28b vector	
SEQ ID NO:95	Reverse primer for <i>BnSCL1</i> ¹⁻¹⁴⁵ for pET-28b vector	
SEQ ID NO:96	Forward primer for <i>BnSCL1</i> , <i>BnSCL1</i> ¹⁻³⁵⁸ , <i>BnSCL1</i> ¹⁻²⁶¹ , <i>BnSCL1</i> ¹⁻²¹⁷ and <i>BnSCL1</i> ¹⁻¹⁴⁵ for pPC86 vector	
SEQ ID NO:97	Forward primer for <i>BnSCL1</i> ¹⁴⁶⁻³⁵⁸ for PC86 vector	
SEQ ID NO:98	Forward primer for <i>BnSCL1</i> ²¹⁸⁻⁴³⁴ for PC86 vector	
SEQ ID NO:99	Reverse primer for <i>BnSCL1</i> and <i>BnSCL1</i> ²¹⁸⁻⁴³⁴ for PC86 vector	
SEQ ID NO:100	Reverse primer for <i>BnSCL1</i> ¹⁻³⁵⁸ for PC86 vector	
SEQ ID NO:101	Reverse primer for <i>BnSCL1</i> ¹⁻²⁶¹ for PC86 vector	
SEQ ID NO:102	Reverse primer for <i>BnSCL1</i> ¹⁻²¹⁷ for PC86 vector	
SEQ ID NO:103	Reverse primer for <i>BnSCL1</i> ¹⁻¹⁴⁵ for PC86 vector	
SEQ ID NO:104	aa seq of LXXLL motif (¹⁴⁸ LGSLL ¹⁵²)	

The above description is not intended to limit the claimed invention in any manner, furthermore, the discussed combination of features might not be absolutely necessary for the inventive solution.

The present invention will be further illustrated in the following examples. However it is to be understood that these examples are for illustrative purposes only, and should not be used to limit the scope of the present invention in any manner.

5 Examples

Materials and Methods

Plant Material

10

Wild type *Arabidopsis thaliana*, ecotype Columbia, seeds were germinated on RediEarth (W.R. Grace & Co.) soil in pots covered with window screens under green house conditions (~25°C, 16 hr light). Emerging bolts were cut back to encourage further bolting. Plants were used for transformation once multiple secondary bolts had
15 been generated.

Plant Transformation

Plant transformation was carried out according to the floral dip procedure
20 described in Clough and Bent (1998). Essentially, *Agrobacterium tumefaciens* transformed with the construct of interest (using standard methods as known in the art) was grown overnight in a 100ml Luria-Bertani Broth (10 g/L NaCl, 10 g/L tryptone, 5 g/L yeast extract) containing 50 µg/ml kanamycin. The cell suspension culture was centrifuged at 3000 X g for 15 min. The pellet was resuspended in 1L of
25 the transformation buffer (sucrose (5%), Silwet L77 (0.05%)(Loveland Industries). The above-ground parts of the *Arabidopsis* plants were dipped into the *Agrobacterium* suspension for ~1 min and the plants were then transferred to the greenhouse. The entire transformation process was repeated twice more at two day intervals. Plants were grown to maturity and seeds collected. To select for transformants, seeds were
30 surface sterilized by washing in 0.05% Tween 20 for 5 minutes, with 95% ethanol for 5 min, and then with a solution containing sodium hypochlorite (1.575%) and Tween 20 (0.05%) for 10 min followed by 5 washings in sterile water. Sterile seeds were plated onto either Pete Lite medium (20-20-20 Peter's Professional Pete Lite fertilizer (Scott) (0.762 g/l), agar (0.7%), kanamycin (50 µg/ml), pH 5.5) or MS medium (MS

salts (0.5X)(Sigma), B5 vitamins (1X), agar (0.7%), kanamycin (50 μ g/ml) pH 5.7). Plates were incubated at 20°C, 16hr light/ 8 hr dark in a growth room. After approximately two weeks, seedlings possessing green primary leaves were transferred to soil for further screening and analysis.

5

Example 1: Optimization of ROS protein coding region.

The *ros* nucleotide sequence is derived from *Agrobacterium tumefaciens* (SEQ ID NO:1; Figure 1A). Analysis of the protein coding region of the *ros* nucleotide sequence indicates that the codon usage may be altered to better conform to plant translational machinery. The protein coding region of the *ros* nucleotide sequence was therefore modified to optimize expression in plants (SEQ ID NO:2; Figure 1B). The nucleic acid sequence of the ROS repressor was examined and the coding region modified to optimize for expression of the gene in plants, using a procedure similar to that outlined by Sardana et al. (1996). A table of codon usage from highly expressed genes of dicotyledonous plants was compiled using the data of Murray et al. (1989). The *ros* nucleotide sequence was also modified (SEQ ID NO:2; Figure 1B) to ensure localization of the ROS repressor to the nucleus of plant cells, by adding a SV40 nuclear localization signal (Rizzo,P. et al., 1999; The nuclear localization signal resides at amino acid positions 126-132; accession number AAF28270).

The *ros* gene is cloned from *Agrobacterium tumefaciens* by PCR. The nucleotide sequence encoding the ROS protein is expressed in, and purified from, *E. coli*, and the ROS protein used to generate an anti-ROS antiserum in rabbits using standard methods (Sambrook et al.).

Example 2: Constructs placing a nucleic acid sequence of interest under transcriptional control of regulatory regions that have been modified to contain ROS operator sites, and preparation of reporter lines.

p74-315: Construct for The Expression of GUS Gene Driven by a CaMV 35S Promoter Containing a ROS Operator Downstream of TATA Box (Figure 5(A)).

The *Bam*HI-*Eco*RV fragment of CaMV 35S promoter in pBI121 is cut out and replaced with a similar synthesized DNA fragment in which the 25 bp immediately downstream of the TATA box were replaced with the ROS operator sequence:

5

TATATTTCAATTTTATTGTAATATA (SEQ ID NO: 7).

Two complementary oligos, ROS-OPDS (SEQ ID NO:28) and ROS-OPDA (SEQ ID NO:29), with built-in *Bam*HI-*Eco*RV ends, and spanning the *Bam*HI-*Eco*RV region of CaMV35S, in which the 25 bp immediately downstream of the TATA box are replaced with the ROS operator sequence (SEQ ID NO: 7), are annealed together and then ligated into the *Bam*HI-*Eco*RV sites of CaMV35S.

ROS-OPDS: 5'-ATC TCC ACT GAC GTA AGG GAT GAC GCA CAA TCC CAC
15 TAT CCT TCG CAA GAC CCT TCC TCT ATA TAA TAT ATT
TCA ATT TTA TTG TAA TAT AAC ACG GGG GAC TCT AGA G-
3' (SEQ ID NO:28)

ROS-OPDA: 5'- G ATC CTC TAG AGT CCC CCG TGT TAT ATT ACA ATA
20 AAA TTG AAA TAT ATT ATA TAG AGG AAG GGT CTT GCG
AAG GAT AGT GGG ATT GTG CGT CAT CCC TTA CGT CAG
TGG AGA T-3' (SEQ ID NO:29)

The p74-315 sequence from the *Eco*RV site (GAT ATC) to the first codon (ATG) of GUS is shown below (TATA box - lower case in bold; the synthetic ROS sequence - bold caps; a transcription start site - ACA, bold italics; *Bam*HI site - GGA TCC; and the first of GUS, ATG, in italics; are also indicated):

5'-GAT ATC TCC ACT GAC GTA AGG GAT GAC GCA CAA TCC CAC TAT
30 CCT TCG CAA GAC CCT TCC TCt ata taA TAT ATT TCA ATT TTA TTG
TAA TAT A*AC* ACG GGG GAC TCT AGA GGA TCC CCG GGT GGT CAG TCC
CTT ATG-3'
(SEQ ID NO:40)

p74-316: Construct for The Expression of GUS Driven by a CaMV 35S Promoter Containing a ROS Operator Upstream of TATA Box (Figure 5(B)).

The *Bam*HI-*Eco*RV fragment of CaMV 35S promoter in pBI121 is cut out and replaced with a similar synthesized DNA fragment in which the 25 bp immediately upstream of the TATA box are replaced with the ROS operator sequence (SEQ ID NO: 7). Two complementary oligos, ROS-OPUS (SEQ ID NO:30) and ROS-OPUA (SEQ ID NO:31), with built-in *Bam*HI-*Eco*RV ends, and spanning the *Bam*HI-*Eco*RV region of CaMV35S, in which the 25 bp immediately upstream of the TATA box were replaced with a ROS operator sequence (SEQ ID NO: 7), are annealed together and then ligated into the *Bam*HI-*Eco*RV sites of CaMV35S.

ROS-OPUS: 5'-ATC TCC ACT GAC GTA AGG GAT GAC GCA CAA TCT ATA
TTT CAA TTT TAT TGT AAT ATA CTA TAT AAG GAA GTT
CAT TTC ATT TGG AGA GAA CAC GGG GGA CTC TAG AG -3'
(SEQ ID NO:30)

ROS-OPUA: 5'- G ATC CTC TAG AGT CCC CCG TGT TCT CTC CAA ATG
AAA TGA ACT TCC TTA TAT AGT ATA TTA CAA TAA AAT
TGA AAT ATA GAT TGT GCG TCA TCC CTT ACG TCA GTG
GAG AT-3' (SEQ ID NO:31)

The p74-316 sequence from the *Eco*RV site (GAT ATC) to the first codon (ATG) of GUS is shown below (TATA box - lower case in bold; the synthetic ROS sequence - bold caps; a transcription start site - ACA, bold italics; *Bam*HI site - GGA TCC; the first codon of GUS, ATG -italics, are also indicated):

5'-GAT ATC TCC ACT GAC GTA AGG GAT GAC GCA CAA TCT ATA TTT
CAA TTT TAT TGT AAT ATA Cta tat aAG GAA GTT CAT TTC ATT TGG
AGA GAA CAC GGG GGA CTC TAG AGG ATC CCC GGG TGG TCA GTC CCT
TAT G-3' (SEQ ID NO:41)

p74-309: Construct for The Expression of GUS Driven by a CaMV 35S Promoter Containing ROS Operators Upstream and Downstream of TATA Box (Figure 5(C)).

5 The *Bam*HI-*Eco*RV fragment of CaMV 35S promoter in pBI121 is cut out and replaced with a similar synthesized DNA fragment in which the 25 bp immediately upstream and downstream of the TATA box were replaced with two ROS operator sequences (SEQ ID NO: 7). Two complementary oligos, ROS-OPPS (SEQ ID NO:32) and ROS-OPPA (SEQ ID NO:33), with built-in *Bam*HI-*Eco*RV ends, and spanning the *Bam*HI-*Eco*RV region of CaMV35S, in which the 25 bp immediately upstream and downstream of the TATA box are replaced with two ROS operator sequences, each comprising the sequence of SEQ ID NO: 7 (in italics, below), are annealed together and ligated into the *Bam*HI-*Eco*RV sites of CaMV35S.

15 ROS-OPPS: 5'-ATC TCC ACT GAC GTA AGG GAT GAC GCA CAA TCT *ATA TTT CAA TTT TAT TGT AAT ATA CTA TAT AAT ATA TTT CAA TTT TAT TGT AAT ATA ACA CGG GGG ACT CTA GAG*-3' (SEQ ID NO:32)

ROS-OPPA: 5'-G ATC CTC TAG AGT CCC CCG TGT TAT ATT ACA ATA AAA TTG AAA TAT ATT ATA TAG *TAT ATT ACA ATA AAA TTG AAA TAT AGA TTG TGC GTC ATC CCT TAC GTC AGT GGA GAT*-3' (SEQ ID NO:33)

20

The **p74-309** sequence from the *Eco*RV site (GAT ATC) to the first codon (ATG) of GUS is shown below (TATA box - lower case in bold; two synthetic ROS sequence - bold caps; a transcription start site - ACA, bold italics; *Bam*HI site - GGA TCC; the first codon of GUS, ATG -italics, are also indicated):

25

5'-GAT ATC TCC ACT GAC GTA AGG GAT GAC GCA CAA TCT ATA TTT CAA TTT TAT TGT AAT ATA Cta tat aAT ATA TTT CAA TTT TAT TGT AAT ATA *ACA* CGG GGG ACT CTA GAG GAT CCC CGG GTG GTC AGT CCC TTA TG-3' (SEQ ID NO:42)

30

p74-118 Construct for The Expression of GUS Driven by a CaMV 35S Promoter Containing three ROS Operators Downstream of TATA Box (Figure 5(D)).

5 The *Bam*HI-*Eco*RV fragment of CaMV 35S promoter in pBI121 is cut out and replaced with a similar synthesized DNA fragment in which a region downstream of the TATA box was replaced with three ROS operator sequences (SEQ ID NO:43). The first of the three synthetic ROS operator sequences is positioned immediately of the TAT box, the other two ROS operator sequence are located downstream of the
10 transcriptional start site (ACA). Two complementary oligos with built-in *Bam*HI-*Eco*RV ends were prepared as describe above for the other constructs. were annealed together and ligated into the *Bam*HI-*Eco*RV sites of CaMV35S.

The **p74-118** sequence from the *Eco*RV site (GAT ATC) to the first codon
15 (ATG) of GUS is shown below (TATA box - lower case in bold; three synthetic ROS sequence - bold caps; a transcription start site - ACA, bold italics; *Bam*HI site - GGA TCC; the first codon of GUS, ATG -italics, are also indicated):

5'-GAT ATC TCC ACT GAC GTA AGG GAT GAC GCA CAA TCC CAC TAT
20 CCT TCG CAA GAC CCT TCC TCT **ata taA** TAT ATT TCA ATT TTA TTG
TAA TAT **AAC ACG** GGG GAC TCT AGA GGA TCC TAT ATT TCA ATT TTA
TTG TAA TAT AGC TAT ATT TCA ATT TTA TTG TAA TAT AAT CGA TTT
CGA ACC CGG GGT ACC GAA TTC CTC GAG TCT AGA GGA TCC CCG GGT
GGT CAG TCC CTT *ATG*-3' (SEQ ID NO:43)

25

p76-508: Construct for The Expression of The GUS Gene Driven by the tms2 Promoter Containing a ROS Operator (Figure 6(B)).

The tms2 promoter is PCR amplified from genomic DNA of *Agrobacterium*
30 *tumefaciens* 33970 using the following primers:

sense primer: 5'-TGC GGA TGC ATA AGC TTG CTG ACA TTG CTA GAA
AAG- 3' (SEQ ID NO:36)

anti-sense primer: 5'-CGG GGA TCC TTT CAG GGC CAT TTC AG- 3'(SEQ ID NO:37)

The 352 bp PCR fragment is cloned into the *EcoRV* site of pBluescript, and sub-cloned into pGEM-7Zf(+). Two complementary oligos, ROS-OP1 (SEQ ID NO:34) and ROS-OP2 (SEQ ID NO:35), containing two ROS operators (in italics, below), are annealed together and cloned into pGEM-7Zf(+) as a *Bam*HI/*Cla*I fragment at the 3' end of the *tms2* promoter. This promoter/operator fragment is then sub-cloned into pBI121 as a *Hind*III/*Xba*I fragment, replacing the CaMV 35S promoter fragment.

ROS-OP1: 5'-GAT CCT ATA TTT CAA TTT TAT TGT AAT ATA GCT *ATA TTT CAA TTT TAT TGT AAT ATA* AT-3' (SEQ ID NO:34)

ROS-P2: 5'-CGA TTA TAT TAC AAT AAA ATT GAA ATA TAG *CTA TAT TAC AAT AAA ATT GAA ATA TAG*-3'(SEQ ID NO:35).

As a control, p76-507 comprising a *tms2* promoter (without any operator sequence) fused to GUS (Figure 4(A)), is also prepared.

p74-501: Construct for The Expression of The GUS Gene Driven by The Actin2 Promoter Containing a ROS operator (Figure 7B)).

The Actin2 promoter is PCR amplified from genomic DNA of *Arabidopsis thaliana* ecotype Columbia using the following primers:

Sense primer: 5'- AAG CTT ATG TAT GCA AGA GTC AGC-3'(SEQ ID NO:38)

SpeI

Anti-sense primer: 5'- TTG ACT AGT ATC AGC CTC AGC CAT-3'(SEQ ID NO:39)

The PCR fragment is cloned into pGEM-T-Easy. Two complementary oligos, ROS-OP1 (SEQ ID NO:34) and ROS-OP2 (SEQ ID NO:35), with built-in *Bam*HI and *Cla*I sites, and containing two ROS operators, are annealed together and inserted into the

Actin2 promoter at the *Bgl*II/*Cla*I sites replacing the *Bgl*II/*Cla*I fragment. This modified promoter is inserted into pBI121 vector as a *Hind*III/*Bam*HI fragment.

As a control, p75-101, comprising an actin2 promoter (without any operator sequence) fused to GUS (Figure 7(A)), is also prepared.

The various constructs are introduced into *Arabidopsis*, as described above, and transgenic plants are generated. Transformed plants are verified using PCR or Southern analysis. Figure 8(A) show Southern analysis of transgenic plants comprising a first genetic construct, for example, p74-309 (35S-ROS operator sequence-GUS, Figure 5(C))

Example 3: Crossing of transgenic lines containing fusion constructs with transgenic lines containing GUS reporter constructs.

Transgenic *Arabidopsis* lines containing fusion constructs (second genetic constructs) are crossed with lines containing appropriate reporter (GUS) constructs (first genetic constructs). To perform the crossing, open flowers are removed from plants of the reporter lines. Fully formed buds of plants of the repressor lines are gently opened and emasculated by removing all stamens. The stigmas are then pollinated with pollen from plants of the repressor lines and pollinated buds are tagged and bagged. Once siliques formed, the bags are removed, and mature seeds are collected. Plants generated from these seeds are then used to determine the level of reporter gene (GUS) repression by GUS staining. Levels of GUS expression in the hybrid lines are compared to those of the original reporter lines. Plants showing a modified GUS expression levels are further characterized using PCR, Southern and Northern analysis.

Example 4: Preparation of a Chromatin Remodelling Factor

HDAC was used as an example of a chromatin remodelling factor that may be isolated from an organism. Transcription factors that recruit histone deacetylase (HDAC) to target promoters in *Brassica napus* were identified *in vivo* by screening a yeast two-hybrid library using the *Arabidopsis thaliana* HDA19 as bait. A cDNA

clone that encodes a novel protein, bnKCP1, containing a kinase-inducible domain (KID) was identified. Southern blot analysis indicated that the *bnKCP1* gene belongs to a small gene family of at least three members, and northern blot analysis showed that it was strongly expressed in stems, flowers, roots and immature siliques seeds, but not in leaf blades. *In vitro* protein binding assays showed that the protein is able to interact with both HDA19 and histone acetyltransferase (HAT) and that the KID domain is required for this interaction with HDA19 and HAT in vitro. When assayed *in vivo*, bnKCP1 exerted modest activation of transcription of a reporter gene in yeast.

The cAMP-responsive element (CRE) binding protein (CREB) binds to the CREB-binding protein (CBP) in response to extracellular stimuli that induce intracellular accumulation of secondary messengers Ca^{2+} and cAMP. The KID domain is highly conserved in the CREB family proteins, CREB, CREM and ATF-1 (Montminy, 1997). Each protein in this family has a serine phosphorylation site (RRPS¹³³) within the KID domain, which is recognised by protein kinase A (PK-A) that phosphorylates S¹³³. PK-A in turn is induced by outside stimuli that induce intracellular accumulation of Ca^{2+} and cAMP. CREB binding activity is regulated through S¹³³ phosphorylation, which leads to interaction of CREB with CBP. The KIX domain of CBP is required for interaction with the KID domain of CREB having a phosphorylated S¹³³ (see review Montminy, 1997). Interestingly, CBP possesses intrinsic HAT activity (Bannister and Kouzarides, 1996; Ogryzko et al., 1996) suggesting that recruitment of CBP to target promoters by the transcription activator CREB may contribute to the transcriptional activation of CRE-dependent genes by the involvement of histone acetylation at the genetic loci of target genes.

In *Arabidopsis*, a HAT gene encoding an ortholog of the yeast GCN5 was found to bind *in vitro* to two proteins similar to the yeast HAT-adaptor proteins ADA2, ADA2a and ADA2b (Stockinger et al., 2001). Moreover, the transcription activator CBF1 was found to bind to both HAT and ADA2, indicating that these proteins might be recruited to target cold-inducible genes by binding to CBF1 (Stockinger et al., 2001). The finding that the *Arabidopsis* ADA2 and GCN5 genes share similarity with their counterparts in yeast and humans suggests that chromatin remodelling complexes are conserved even among evolutionary distant organisms.

Experimental Procedures

Brassica napus L. cv Cascade (winter type), Westar (spring type) and DES010 (spring type) were used for the isolation of genomic DNA and total RNA. Leaves, flowers, stems, siliques and immature seeds were harvested from plants cultured in a controlled-environment greenhouse programmed for a photoperiod of 16h day and 8h night. Roots were obtained by culturing sterilized seeds in 0.8% agar plates containing ½ MS medium and 1% sucrose. For cold acclimation (4°C), abscisic acid (250 µM), drought and high salt (850 mM NaCl) treatments, four-leaf stage seedlings were treated and fourth fully expanded leaf blades were harvested as described by Gao et al. (2002). LaCl₃ and inomycin treatments were carried out by watering four-leaf stage plants with 20 mM LaCl₃ and 10 µM inomycin, respectively. Plants were covered with Saran Wrap to slow evaporation.

Yeast two-hybrid screening and cloning

A yeast two-hybrid cDNA library (Dr. Isobel Parkin, Agriculture and Agri-Food Canada Research Centre, Saskatoon) was constructed from poly(A) mRNA isolated from the above-ground parts of the four-leaf stage seedlings of *B. napus* L. cv. DH12075 and cloned into a GAL4 AD (activation domain) vector pPC86 using the SuperScript Plasmid System for cDNA Synthesis and Plasmid Cloning (GibcoL BRL).

To generate the pDBLeu-HDA19 construct, the entire coding region of *Arabidopsis thaliana* RPD3-type HDA19 cDNA (Accession # AF195547) was PCR amplified using PWO DNA polymerase (Roche) with a forward primer:

5'-GCGTCGACGATGGATACTGGCGGCAATTCGC-3' (SEQ ID NO: 44)

and a reverse primer:

5'-AGGCGGCCGCTTATGTTTTAGGAGGAAACGCC-3'. (SEQ ID NO: 45)

The identity of the PCR product was confirmed by DNA sequence analysis and inserted into the *SalI* and *NotI* sites of the Gal4 DB (DNA binding domain) vector pDBLeu in-frame with the GAL4 sequence and used as a bait to screen the *B. napus* cDNA library using PROQUEST Two-Hybrid System (GibcoL BRL).

5

Approximately 1×10^6 transformants were subjected to the two-hybrid selection on synthetic complete (SC) medium lacking leucine, tryptophan and histidine but containing 15 mM 3-amino-1,2,4-triazole (3AT®). The expression of the *HIS3* reporter gene allowed colonies to grow on the selective medium and the putative

10 His⁺ (3AT®) positive transformants were tested for the induction of the two other reporter genes, *URA3* and *lacZ*. The positive colonies were reassessed by retransformation assays and the cloned cDNAs were identified by PCR and DNA sequence analysis.

15 Southern blot analysis

Total genomic DNA was isolated from the leaves of *B. napus* L. cv Westar using a modified CTAB (cetyltriethylammonium bromide) extraction method (Gao et al., 2002). Briefly, 10 µg of total genomic DNA was digested with *EcoRI*, *XbaI*,

20 *HindIII*, *PstI*, *EcoRV* and *KpnI* restriction endonucleases, separated on a 0.8% agarose gel, transferred to Hybond-XL membranes (Amersham Pharmacia) and hybridized with the *bnKCP1* open reading frame (ORF) labeled with [α -³²P]dCTP using random primer labeling procedure. The DNA fragment to be used as a probe was isolated from a 0.8% agarose gel and purified with a QIAquick Gel Extraction

25 Kit (Qiagen), and the probe was purified with a ProbeQuant G-50 Micro Column (Amersham Pharmacia). Hybridization was performed under high stringency conditions (Gao, M.-J. et al., 2002).

Northern blot analysis

30

Total RNA was isolated from the tissues of *B. napus* L. cv DES010. These included leaves and stems of four-leaf stage seedlings, flowers, immature siliques of adult plants, and roots of cultured seedlings as described by Gao et al. (2001). Probe

labelling, hybridization, washing and membrane stripping were performed as described above in the Southern blot analysis Section.

Expression and purification of recombinant Gcn5 and HDA19

5

The full coding regions of the *Arabidopsis* HAT, Gcn5 (Dr. M. Thomashow, Michigan State University, MI), and HDA19 (Accession # AF195547) were PCR amplified, sequence analyzed and inserted in-frame with the GST (glutathione s-transferase) into the *Sa*I and *Not*I sites of vector pGEX-6P-2 (Amersham Pharmacia). The forward used for the amplification of Gcn5 was:

10

5'- GCGTCGACGATGGACTCTCACTCTTCCCACC-3' (SEQ IDNO: 46)

and the reverse primer for Gcn5 was:

15

5'-GCGCGGCCCGCCTATTGAGATTTAGCACCAGA-3' (SEQ IDNO: 47)

20

The forward primer for HDA19 was SEQ ID NO: 44, as listed above, and the reverse primer was:

5'-GCGCGGCCCGCTTATGTTTTAGGAGGAAACGC-3' (SEQ ID NO:48).

25

Recombinant pGEX-6P-2 plasmids were used to transform *E. coli* BL21-CodonPlus (DE3)-RP competent cells (Stratagene). Expression and purification under non-denaturing conditions were carried out as described by Gao et al. (Gao, M.-J. et al., 2002). The GST-Gcn5 and GST-HDA19 fusion proteins were analyzed by 7.5% SDS-PAGE (SDS-polyacrylamide gel electrophoresis) and western blotting with rabbit anti-GST-Pi polyclonal antibody (Chemicon) using ECL Western blotting analysis system (Amersham Pharmacia).

30

Generation of deletion mutants of bnKCP1

The two fragments, *bnKCP1*¹⁻¹⁶⁰ and *bnKCP1*¹⁻⁸⁰, and the entire coding region of *bnKCP1* DNA encoding amino acids 1-80, 1-160 and 1-215, respectively, were amplified by PCR and cloned into the *Hind*III and *Xho*I sites of pET-28-b vector (Novagen, Madison, WI). The primers used for the amplification were as follows:

5 *bnKCP1*¹⁻¹⁶⁰ (240bp): forward primer:

5'-GCAAGCTTATGGCAGGAGGAGGACCAACT-3' (SEQ ID NO:49),

reverse primer:

10

5'-CGCTCGAGCTCCTCCTCATCATTGTCTTC-3' (SEQ ID NO:50);

*bnKCP1*¹⁻⁸⁰ (480bp): forward primer:

15 5'-GCAAGCTTATGGCAGGAGGAGGACCAACT-3' (SEQ ID NO:49),

reverse primer

5'- CGCTCGAGATGAACAGGCAAAAGAGGCAT-3' (SEQ ID NO:51);

20

bnKCP1 (645bp): forward primer:

5'-GCAAGCTTATGGCAGGAGGAGGACCAACT-3'(SEQ ID NO:49),

25 reverse primer

5'- CGCTCGAGCTCaTCTTCTTCTTCTTCTTC-3' (SEQ ID NO:52).

In vitro protein interaction assays

30 Full-length *bnKCP1* and truncated mutant *bnKCP1*¹⁻¹⁶⁰ and *bnKCP1*¹⁻⁸⁰ proteins labeled with [³⁵S]methionine were produced using TNT-Quick Coupled Transcription/Translation System (Promega) according to the manufacture's instructions, with some modifications. A total of 1 μ l of RNase inhibitor (GibcoL BRL) and 1 μ l of protease inhibitors set (Roche) were added to the lysate reaction.

After incubation for 90 min at 30°C, RNase A was added to the reaction to a final concentration of 0.2 mg/ml and incubated for 5 min at the same temperature.

In vitro protein interaction was detected with GST pulldown affinity assays as described by Ahmad et al. (1999) with some modifications. Briefly, 6 µg of GST or 4 µg of GST-fusion protein was incubated with 5 µl of [³⁵S]Met-labeled translation mixture in 200 µl of bead-binding buffer (50 mM K-phosphate, pH 7.6, 450 mM KCl, 10 mM MgCl₂, 10% glycerol, 1% Triton X-100, 1% BSA and 1 µl of diluted 1:12 protease inhibitors set) for 1h at room temperature. After incubation, 20 µl of 50% slurry of glutathione-Sepharose beads containing 10 mg/ml of BSA and 4 µg of EtBr was mixed with the reaction mixture followed by gentle rotation for 1h at 4°C. After washing six times with 1.2 ml of bead-binding buffer without BSA and EtBr but containing 12 µl of protease inhibitors set (Roche), the bound proteins were eluted with 30 µl of 2 X SDS loading buffer, boiled for 2 min and analyzed by 12% SDS-PAGE. After electrophoresis, the gels were dried, treated with Amplify (Amersham Pharmacia) and subjected to fluorography.

In vivo protein assays

The entire region of *bnKCP1* and the two fragments, *bnKCP1*¹⁻¹⁶⁰ and *bnKCP1*¹⁻⁸⁰, were PCR amplified and cloned into the *Sa*II and *Not*I sites of pPC86 vector (GibcoL BRL) in-frame with the GAL4 AD sequences to generate constructs pPC86-*bnKCP1*, pPC86-*bnKCP1*¹⁻¹⁶⁰ and pPC86-*bnKCP1*¹⁻⁸⁰. The oligonucleotide primers used in PCR amplification were as follows:

bnKCP1, *bnKCP1*¹⁻¹⁶⁰ and *bnKCP1*¹⁻⁸⁰ forward primer

5'- GCGTCGACGATGGCAGGAGGAGGACCAACT-3' (SEQ ID NO:53)

bnKCP1 reverse primer

5'- GCGCGGCCGCGCCTCATCTTCTTCTTCTCCTC -3' (SEQ ID NO:54)

*bnKCP1*¹⁻¹⁶⁰ reverse primer

5'- GCGCGGCCGCGCATGAACAGGCAAAAGAGGCAT -3' (SEQ ID NO:55)

*bnKCP1*¹⁻⁸⁰ reverse primer

5'- GCGCGGCCGCTCCTCCTCATCATTGTCTTC-3' (SEQ ID NO:56)

For in vivo protein interaction assays, the MaV203 yeast cells carrying the reporter gene *lacZ* and the construct pDBLeu-HDA19, in which the HDA19 was fused in-frame with GAL4DB, were transfected with either of the plasmids pPC86-*bnKCP1*, pPC86-*bnKCP1*¹⁻¹⁶⁰ and pPC86-*bnKCP1*¹⁻⁸⁰ or the vector alone. The expression of *lacZ* reporter gene was quantified by measuring the β -galactosidase activity using chlorophenol red- β -D-galactopyranoside (CPRG) according to the manufacturer's instructions (GibcoL BRL). Two yeast control strains A and B (GibcoL BRL) were used as negative and positive controls, respectively.

Site-directed mutagenesis (SDM)

The QuickChange site-directed mutagenesis kit (Stratagene) was used to replace the serine residue in the PK-A phosphorylation site (RRPS¹⁸⁸) within the KID domain with a glycine residue to generate *bnKCP1G*¹⁸⁸ according to the manufacturer's instructions. The two oligonucleotide primers used in SDM were as follows:

20

forward primer:

5'- GATGTTCTTGCGAGGAGACCAGGATTCAAGAACAGAGCATTGAAG-3'
(SEQ ID NO:57)

25 reverse primer:

5'- CTTCAATGCTCTGTTCTTGAATCCTGGTCTCCTCGCAAGAACATC-3'
(SEQ ID NO:58)

The introduced mutation was confirmed by DNA sequencing, and the mutated *bnKCP1G*¹⁸⁸ was cloned into the *HindIII* and *XhoI* sites of pET-28b vector to generate pET-*bnKCP1G*¹⁸⁸, which was then used for in vitro protein interaction assays as described above.

30

Transactivation assay using yeast one-hybrid system

Effector plasmids pDBLeu-bnKCP1¹⁻¹⁶⁰, pDBLeu-bnKCP1¹⁻⁸⁰, pDBLeu-bnKCP1⁸¹⁻²¹⁵ and pDBLeu-bnKCP1 were constructed by ligating the PCR-amplified fragments Δ bnKCP1¹⁻¹⁶⁰, Δ bnKCP1¹⁻⁸⁰, Δ bnKCP1⁸¹⁻²¹⁵ and the coding region of bnKCP1 into the *SalI/NotI* sites of pDBLeu vector (GibcoL BRL) in-frame with the GAL4 DB sequence. The oligonucleotide primers for PCR amplification were as follows:

10 *bnKCP1* forward primer

5'-GCGTCGACGATGGCAGGAGGAGGACCAACT-3' (SEQ ID NO: 53),

bnKCP1 reverse primer

5'-GCGCGGCCGCGCCTCATCTTCTTCTTCTCCTC-3' (SEQ ID NO:54)

15

*bnKCP1*¹⁻¹⁶⁰ forward primer

5'-GCGTCGACGATGGCAGGAGGAGGACCAACT-3' (SEQ ID NO: 53),

*bnKCP1*¹⁻¹⁶⁰ reverse primer

20 5'- GCGCGGCCGCGCATGAACAGGCAAAAGAGGCAT -3' (SEQ ID NO:55)

*bnKCP1*¹⁻⁸⁰ forward primer

5'-GCGTCGACGATGGCAGGAGGAGGACCAACT-3' (SEQ ID NO: 53),

25 *bnKCP1*¹⁻⁸⁰ reverse primer

5'- GCGCGGCCGCGCCTCCTCCTCATCATTGTCTTC -3' (SEQ ID NO:56)

*bnKCP1*⁸¹⁻²¹⁵ forward primer

5'-GCGTCGACGCTAGGGTTGGCTTCATTGAGA-3' (SEQ ID NO:59)

30

*bnKCP1*⁸¹⁻²¹⁵ reverse primer

5'-GCGCGGCCGCGCCTCATCTTCTTCTTCTCCTC-3' (SEQ ID NO:54)

The three reporter genes, *lacZ*, *HIS3* and *URA3*, which were chromosomally integrated in the genome of MaV203 yeast cells were driven by unrelated promoters containing GAL4 DNA binding sites (GibcoL BRL). For transient assays, the effector constructs or the negative control vector pDBLeu were transferred to the MaV203 yeast cells. The β -galactosidase activity was measured using CPRG (chlorophenol red- β -D-galactopyranoside) according to the manufacturer's instructions (GibcoL BRL). The MaV203 cells containing plasmids pDBLeu-HDA19 and pPC86-bnKCP1 were used as the positive control. In addition, we used three yeast control strains A, B, and C (GibcoL BRL), which were developed to contain plasmid pairs expressing fusion proteins with none, weak and moderately strong protein-protein interaction strength, respectively.

Transient expression of the GUS-bnCKP1 fusion protein

The oligonucleotide primers for PCR amplification of the entire coding region of *bnKCP1* were as follows:

forward primer

5'-GCGAATTCATGGCAGGAGGAGGACCAACT-3' (SEQ ID NO:60),

reverse primer

5'-CGGAGCTCCTCaTCTTCTTCTTCTTCTTC-3' (SEQ ID NO:61).

The amplified sequence was cloned into the *EcoRI* and *ScaI* sites of the binary vector p79-637, a derivative of the vector CB301, to generate construct p77-132, which contains GUS-bnKCP1 fusion under control of the CaMV35S promoter. The onion epidermal layers were transformed with *Agrobacterium* culture prepared as described by Kapila et al (1997) with a few modifications. Briefly, the onion inner epidermal layers were peeled, placed into a culture of *Agrobacterium tumefaciens* strain GV3101 pMP90 containing either p79-637, for GUS expression only, or p77-132 and subjected to continuous vacuum of -85 kPa for 20 min. After incubation at 22°C under 16h light condition for 7 days the tissues were placed into GUS staining solution [100 mM potassium phosphate buffer (pH 7.4), 1 mM EDTA, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, 0.1% Triton X-100, 1 mM 5-bromo-4-chloro-3-

indolyl- β -D-glucuronide (X-gluc)], vacuum infiltrated for 20 min at -85 kPa and incubated overnight at 37°C. To determine the intercellular location of nuclei, tissues were stained with the nucleus-specific 4',6-diamidino-2-phenylindole (DAPI) solution (14 μ g/ml DAPI, 0.1 x PBS, 90% glycerol) (Varagona et al, 1991) and viewed under a
5 Zeiss microscope using both fluorescence and bright-field optics.

Cloning of the *B. napus* KCP protein

To identify proteins that interact with HDA19 in *B. napus*, the ORF of
10 *Arabidopsis* HDA19 fused to the yeast Gal4 DNA binding domain was used as bait in a yeast two-hybrid screening of a *B. napus* cDNA library linked to the yeast Gal4 activation domain. Several positive clones were obtained on the basis of the induction of three yeast reporter genes, *HIS3*, *URA3* and *lacZ* and DNA sequence analysis. One of these clones (963 bp), pPC86-bnKCP1, encodes a 23.5 kDa protein that contains a
15 putative kinase-inducible domain (KID)-like motif, and hence was designated *bnKCP1* (*B. napus* KID-containing protein 1).

Alignment of deduced amino acid sequences indicated that bnKCP1 shares an 82% amino acid identity with atKCP, an *Arabidopsis* unknown 26.6 kDa protein
20 (AY088175, At5g24890). It also shares high similarity in the conserved region of approximately 55 amino acids (GKSKS domain) with other two other atKCP-like *Arabidopsis* unknown proteins, atKCL1 (CAB45910, At4g31510) and atKCL2 (AAD23890, At2g24550) (Figure10A).

25 To estimate the *bnKCP1* gene copy number in *Brassica napus* we carried out Southern blot analysis on of total genomic DNA digested with restriction endonucleases using the entire open reading frame of *bnKCP1* for probing under high stringency conditions (Figure. 12). Digestion with *EcoRI* (EI), *HindIII* (H), *PstI* (P), *EcoRV* (EV) and *KpnI*, none of which has a cutting site within *bnKCP1*, resulted in
30 the detection of three bands, whereas digestion with *XbaI* generated six bands, because of the existence of an internal cutting site for *XbaI* in the *bnKCP1* gene. This result indicates that *bnKCP1* belongs to a small gene family of three members in the *Brassica. napus* genomes.

Structural features of the bnKCP1 protein

The ORF of *bnKCP1* gene codes for a 215 amino acid polypeptide product of polypeptide with several functional motifs (Figure 11). Based on a search of protein localization sites using PSORT program (<http://psort.nibb.ac.jp>; Nakai and Kanehisa, 1992), bnKCP1 appears to be is a nuclear protein containing a pat7 nuclear localization signal (NLS) PLNKKRR (SEQ ID NO: 62; Figure 10A, residues 127-133). Three acidic motifs (I, II and III) and a serine-rich (S-rich) region (residues 34-58) may function in transcription activation by bnKCP1 (Johnson et al., 1993). The charged motif GKSKS (residues 88-143), which is conserved in all four protein orthologs (Fig. 10A), is rich in basic residues and encompasses the NLS. This suggests that this domain serve the may function of a DNA-binding motif (Figure 11). In addition, bnKCP1 is extremely hydrophilic (Figure 11) suggesting bnKCP1 is an active element in the nuclear matrix.

Amino acid sequence analysis also revealed that bnKCP1 has a KID-like motif (residues 161-215, Figure 10A) with alpha structure at its C-terminal region (Figure 11). The KID is highly conserved in mammalian CREB protein family and functions in transactivation and protein binding (Montminy et al., 1997). The KID in bnKCP1 has a high similarity to the CREB family member ATF-1 (Figure 10B, C) and contains a protein kinase A (PK-A) phosphorylation site (RRPS) that is conserved in the CREB family of proteins (Figure 10B).

Interaction of bnKCP1 with HDA19 and Gcn5

To confirm the interaction detected in the yeast two-hybrid system between the bnKCP1 protein and HDA19, GST pulldown assays were performed using *in vitro* translated bnKCP1 labeled with [³⁵S]Methionine. The bnKCP1 protein was tested for its ability to interact with recombinant GST-HDA19 or GST-Gcn5 fusions expressed in *E. coli*.

As shown in Figure 13B, bnKCP1 bound to both GST-HDA19 and GST-Gcn5 fusion proteins, but not to GST alone. To reassess the interaction of bnKCP1 with Gcn5 *in vivo*, the ORF of the *Arabidopsis* Gcn5 was fused to the yeast Gal4 DNA

binding domain in pDBLeu vector and then used to transform yeast MaV203 cells expressing *bnKCP1* fused to the yeast Gal4 activation domain in pPC86 vector. The transformants showed induction of the three reporter genes, *HIS3*, *URA3* and *lacZ* at a relatively lower level when compared with the induction levels in transformants with *bnKCP1* and HDA19 (data not shown). This result suggests that *bnKCP1* has a preference for binding to HDA19 *in vivo*.

To map the protein binding domain of the *bnKCP1* protein, two C-terminal truncated mutants of *bnKCP1* lacking the KID domain were constructed. These are $\Delta\text{bnKCP1}^{1-160}$ (residues 1-160) and $\Delta\text{bnKCP1}^{1-80}$ (residues 1-80) as shown in Figure 13A. These truncated mutants were assayed for *in vitro* interaction with the recombinant GST-HDA19 or GST-Gcn5 fusion proteins. The two mutant proteins, $\Delta\text{bnKCP1}^{1-160}$ and $\Delta\text{bnKCP1}^{1-80}$, exhibited no interaction with either GST-HDA19 or GST-Gcn5 indicating that the KID domain of *bnKCP1* protein is essential for binding to HDA19 and Gcn5.

The importance of the KID domain for protein binding was also determined *in vivo* using the yeast two-hybrid system. MaV203 yeast cells were co-transformed with pDBLeu-HDA19, and either pPC86-*bnKCP1*, pPC86- $\Delta\text{bnKCP1}^{1-160}$, pPC86- $\Delta\text{bnKCP1}^{1-80}$ or pPC86 alone (Figure 13C). β -galactosidase activity was reduced by at least 50% when pDBLeu-expressing cells were transformed with plasmids expressing either $\Delta\text{bnKCP1}^{1-160}$ or $\Delta\text{bnKCP1}^{1-80}$, both of which lacked KID, as compared to the full-length *bnKCP1*. This finding demonstrates that KID is critical for *bnKCP1* interaction with HDA19 *in vivo*.

To investigate the importance of S¹⁸⁸ for *bnKCP1* interaction with HDA19, the S¹⁸⁸ residue in *bnKCP1* was mutated to G¹⁸⁸ using site-directed mutagenesis to obtain *bnKCPG*¹⁸⁸ protein (Fig 14). This mutated protein was then tested for binding to HDA19 *in vitro*. When compared to *bnKCP1*, the mutated protein, *bnKCPG*¹⁸⁸, has significantly reduced binding to HDA19 (Fig 14). This confirms that S¹⁸⁸ is essential for optimal interaction between *bnKCP1* and HDA19.

Expression pattern of the *bnKCP1* gene

The expression pattern of the *bnKCP1* gene was analyzed by Northern blot analysis of total RNA extracted from various organs of *B. napus*. As shown in Figure 15, two transcripts of similar sizes appear to hybridize to *bnKCP1*, indicating the existence of two homologs of *bnKCP1* mRNAs in *B. napus*. These transcripts accumulated at high levels in flowers, roots, stems and immature siliques, and at low levels in leaves with petioles, but were undetectable in leaf blades (Figures 15, 16).

To investigate the pattern of *bnKCP1* expression in response to environmental stress conditions, total RNA was isolated from leaf blades of four-leaf stage *B. napus* seedlings that were exposed to low temperature (4°C), drought, high salt (NaCl), and ABA treatment, and used for northern blot analysis using a *bnKCP1* probe. Transcripts of both *bnKCP1* homologs accumulated in leaves in response to cold treatment. The lower size (~0.9 kb) transcript appears to be induced within 4h of cold treatment and about 4h earlier than the higher molecular weight (1.1kb) one (Figure 16A). The *bnKCP1* transcript appears to accumulate in response to low temperature (4°C), but expression was not detected in leaf blades of plants grown under drought condition for up to 4 days, high salt stress for up to 11 days, or upon exogenous application of ABA for up to 8 hours (data not shown). Expression of *bnKCP1* in the stems, was repressed upon cold treatment (Figure 16A), suggesting the response of *bnKCP1* transcript to low temperature or the recruitment of HDA19 and HAT to the promoters of cold responsive genes is organ specific.

Since cold acclimation is known to be associated with elevated levels of intracellular concentrations of Ca^{2+} , tests to determine whether Ca^{+2} has any effect on *bnKCP1* expression were performed. Northern blot analysis was performed using total RNA isolated from leaves of seedlings treated with Ca^{2+} channel blocker LaCl_3 and the Ca^{2+} ionophore inomycin at room temperature. Induction of *bnKCP1* expression upon treatment with inomycin was rapid (2 hrs) but short-lived. The *bnKCP1* transcript was undetectable in leaves of seedlings treated with the LaCl_3 (Figure 16B).

Transcription activation by *bnKCP1*

To determine whether bnKCP1 functions as a transcription activator, transactivation experiments were carried out in yeast. A yeast strain carrying three reporter genes, *lacZ*, *HIS3* and *URA3*, driven by promoters fused to GAL4 DNA binding sites and independently integrated into the yeast genome were transfected with the effector plasmid pDBLeu-bnKCP1 comprising *bnKCP1* fused to the GAL4 DB under the control of ADH promoter. The effector stimulated β -galactosidase activity about 8-fold relative to either GAL4 DB alone or yeast control strain A that contains plasmid pairs expressing fusion proteins without protein-protein interaction. A similar result was obtained when the yeast cells were co-transformed with the positive control plasmids pDBLeu-HDA19 and pPC86-bnKCP1 identified by the two-hybrid selection (Figure 17A). Reporter genes *HIS3* and *URA3* were also modestly activated by bnKCP1 (data not shown). Based on these findings, it can be concluded that bnKCP1 exerts transactivation of target genes in *Brassica napus*.

These data demonstrate the isolation of a plant protein that contains a putative KID domain, which interacts with both GCN5 (HAT) and HDA19. *bnKCP1* was highly expressed in all organs tested, except leaf blades, where it was induced in response to cold acclimation, which also resulted in repressing its expression in stems. Furthermore, bnKCP1 exerts transcription activation of a reporter gene when tested in yeast, indicating the function of bnKCP1 as a transcription factor.

To map the transactivation domain of the bnKCP1 protein, one N-terminal truncated mutant of bnKCP1, Δ bnKCP1⁸¹⁻²¹⁵, and two C-terminal truncated mutants, Δ bnKCP1¹⁻¹⁶⁰ and Δ bnKCP1¹⁻⁸⁰ (Fig 17B) were generated and used in *in vivo* transactivation assays in yeast. As shown in Fig 17C, deletion of the KID or GKSKS domains had no significant influence on β -galactosidase activity, whereas deletion of the N-terminus resulted in approximately 65% reduction in β -galactosidase activity.

Nuclear localization of the bnKCP1 protein

Structural and functional analyses showed bnKCP1 to have features typical of transcription factors. To confirm that bnKCP1 is a nuclear proteins, onion epidermal cell layers were transformed with constructs for the expression of either a GUS-bnKCP1 fusion or GUS alone (Fig 18). Using an Agrobacterium-mediated

transformation method (Kapila et al, 1997). As shown in Figure 18, GUS activity was visualized exclusively in the cytoplasm of control onion cell layers. In contrast, a blue precipitate was localized in the nuclei of cell layers transformed with GUS-bnKCP1 fusion construct, although there was still a certain amount of cytoplasm staining, indicating that at least some targeting to the nucleus occurs with the fusion protein.

Expression of *bnKCP1* is organ-specific

The *bnKCP1* gene appears to be part of a multigene family of three members based on Southern blot hybridization. Northern blot analyses showed that two members of this gene family are of similar transcript sizes and expression patterns. This is consistent with information about bnKCP1 orthologs in *Arabidopsis*, where there are one *atKCP* (At5g24890) and two *atKCP-like* members (At4g31510 and At2g24550) of similar sizes ranging from 1kb to 1.2kb. Northern blot analysis revealed that *bnKCP1* mRNA was expressed in flowers, roots, stems and immature siliques (Figure 14). The transcript accumulation, however, was undetectable in leaf blades of *B. napus* seedlings, suggesting tissue/organ-specific expression of the *bnKCP1* gene. However, cold treatment induced bnKCP1 expression in leaves, but repressed it in stems.

The KID domain is conserved in *bnKCP1*

Structural analysis of the bnKCP1 protein revealed that it was a strongly hydrophilic protein (23.5 kDa, pI 4.2) and had characteristic features of a transcription factor, including a putative nuclear localization signal (NLS), a putative basic DNA binding domain, putative acidic activation domains and a protein-protein interaction domain.

An important structural feature of bnKCP1 is the presence of a putative kinase-inducible domain (KID) with alpha secondary structure at the C-terminal region. The KID domain was first identified in mammalian CREB family members CREB, CREM and ATF-1. The KID domain in mammalian CREB is involved in at least two functions, interaction with CBP/p300 and the site for protein kinase A (PK-A) phosphorylation of S¹³³ (Montminy et al., 1997; Gonzalez et al., 1991; Quinn,

1993; Chrivia *et al.*, 1993; Shaywitz *et al.*, 2000). Similar to its counterpart in CREB, which is involved in protein binding, the KID domain of bnKCP1 is required for binding to both HDA19 and GCN5 *in vitro* and *in vivo*. The ability of bnKCP1 to interact with HDA19 indicates that bnKCP1-mediated transcription control requires
5 direct or indirect recruitment of these transcription regulators to promoter regions of target genes regulated by bnKCP1.

Phosphorylation of CREB at Ser¹³³ is required for the interaction of CREB via its KID with CBP and for CREB to activate transcription in response to some
10 extracellular stimuli (Gonzalez *et al.*, 1989; Chrivia *et al.*, 1993). The KID domain in bnKCP1 also contains a putative PK-A phosphorylation site (RRPS¹⁸⁸), which corresponds to the RRPS¹³³ in mammalian CREB.

Intracellular level of Ca⁺² affect *bnKCP1* expression

15 In mammalian cells, outside stimuli that increase intracellular concentrations of Ca²⁺ or cAMP induce the expression of not only PK-A, but also the *CREB* gene (Meyer *et al.*, 1993). Therefore, tests to determine whether conditions that increase intracellular concentrations of Ca²⁺ would induce *bnKCP1* expression were done. *B.*
20 *napus* seedlings were subjected to one of two treatments, cold or ionomycin. Cold acclimation is known to increase intracellular Ca²⁺ concentrations (Monroy and Dhindsa, 1995; Knight *et al.*, 1996), and ionomycin is a known calcium ionophore that increases Ca²⁺ influx (Hurley *et al.*, 1996). These treatments resulted in the induction of *bnKCP1* expression to varying degrees (Figure 16), which indicated that *bnKCP1*
25 is induced by high intracellular Ca⁺² concentrations.

These results suggest a molecular mechanism by which *bnKCP1* functions as a transcription factor to regulate gene expression by recruiting HDAC to the promoter regions of target genes.

30

Example 5: Characterization of the recruitment factor SCL1 and its interaction with the chromatin remodelling factor HDA19

To search for transcription factors additional that recruit histone deacetylase (HDAC) to target promoters in *Brassica napus*, a yeast two-hybrid library was screened using the *Arabidopsis thaliana* HDA19 as bait. This screening resulted in the isolation of a cDNA clone that encodes a SCARECROW-like protein, BnSCL1, which contains a number of putative functional motifs typical of the GRAS family of transcription factors. Southern blot analysis indicated that the *BnSCL1* gene belongs to a small gene family of about three members. *In vitro* and *in vivo* protein interaction assays revealed that BnSCL1 interacts physically with HDA19 through the VHID domain. BnSCL1 also exerted strong transactivation of the *lacZ* reporter gene in yeast, and both N- and C-terminal regions are critical for the transient expression. Quantitative RT-PCR and RNA gel blot analysis showed that *BnSCL1* was expressed at relatively high level in roots, moderate level in flowers, weak in mature leaves and stems, and barely detectable in immature siliques. The accumulation of *BnSCL1* transcript was regulated by 2,4-D in shoots, roots and matured leaves. Furthermore, the response of *BnSCL1* to 2,4-D was modulated by histone deacetylase HDA19. These results strongly suggest a molecular mechanism by which BnSCL1 functions as a transcription factor to regulate gene expression by recruiting HDAC to the promoter regions of auxin-responsive genes.

20 Plant materials

Brassica napus L. cv. DH12075 was used for DNA and total RNA isolation. Leaves, flowers, stems, siliques and immature seeds were harvested from plants cultured in a controlled-environment greenhouse programmed for a photoperiod of 16h day and 8h night. Roots were obtained by culturing sterilized seeds in 0.8% agar plates containing ½ MS medium (Murashige and Skoog, 1962) and 1% sucrose.

Tissue treatment

30 In exogenous applied auxin treatments, four-leaf stage seedlings grown at 20°C were treated with a foliar spray containing 1mM 2,4-D and 50 mM sodium phosphate, pH 7.5. The four leaves were collected at 30 min, 60 min and 180 min after the first foliar application of 2,4-D. For the measurement of response of shoots and roots to auxin, sterilized seeds were germinated on plates in a growth chamber

with continuous light at 20°C, and 10 dpg seedlings were supplied with varied concentration of 2,4-D. In the auxin transport inhibition experiments, 9 dpg seedlings were incubated in the medium supplemented with 50 µM NPA dissolved in 0.1% DMSO for 24 h before the 2,4-D treatment. For the HDAC inhibitor treatments, 10 mM sodium butyrate was added onto the growth medium and incubated for 24 h followed by exogenous 2,4-D application at varied concentrations.

Yeast two-hybrid screening and cloning

10 A yeast two-hybrid cDNA library was constructed from seedlings of *B. napus* L. cv. DH12075 and screened using a *Arabidopsis thaliana* RPD3-type HDAC (*HDA19*) as bait, with the methods of PROQUEST Two-Hybrid System (GibcoL-BRL) as previously described by Gao *et al.* (2003). The positive colonies were reassessed with retransformation experiments and confirmed with *in vitro* protein interaction
15 assays, and the cloned cDNAs were identified by PCR and DNA sequence analysis.

Gel Blot Analysis

Total genomic DNA was extracted from the leaves of four-leaf stage *B. napus* using a modified CTAB (cetyltriethylammonium bromide) extraction method, and
20 DNA gel blots were prepared and hybridized with the *BnSCL1* open reading frame labeled with [α -³²P]dCTP using random primer labeling procedure as described by Gao *et al.* (2003). Total RNA was isolated using hot phenol method with the first extraction for 30 sec at 80°C as previously described (Gao *et al.* 2002). RNA was
25 isolated from various tissues, including leaves and stems of four-leaf stage seedlings, flowers, immature seeds and siliques of adult plants, and roots of cultured seedlings.

Quantitative RT-PCR

30 Total RNA extracted as described above was treated with Amplification Grade Deoxyribonuclease I (GibcoL BRL) following the manufacture's instructions. The RNA samples were then directly used for reverse transcription prior to amplification without purification. The RT-PCR was quantitatively performed and completed in a

one-step reaction using Superscript One-Step RT-PCR System (GibcoL BRL) as described by Gao *et al.* (2002). Gene-specific sense and anti-sense primers used to generate a 960 bp fragment of *Brassica napus Actin*, as an internal standard, were as described in Gao *et al.*, (2002). Gene-specific primers for the generation of *BnSCL1*,
5 *BnIAA1* and *BnIAA12* fragments were as follows:

BnSCL1 (435 bp)

sense: 5'-GATGGACGAACATGCCATGCGTTCCA-3' (SEQ ID NO:84)

anti-sense: 5'-CGCTCGGATCTTCTGAACAAT-3' (SEQ ID NO:85)

10

BnIAA1 (537bp)

sense: 5'- CCACGCGTCCGGTACGATGAT-3' (SEQ ID NO:86)

anti-sense: 5'- GAAGTTGAGAAATGGTTTATGA-3' (SEQ ID NO:87)

15 *BnIAA12* (659bp)

sense: 5'- ACGCTGGTGCTTCTCCTCCTC-3' (SEQ ID NO:88)

anti-sense: 5'- AAAACCCATTAGAAGAACCAAGAA-3' (SEQ ID NO:89)

20 *BnIAA1* and *BnIAA12* are clones ML2798 and ML4744, which are homologs of *Arabidopsis IAA1* and *IAA12*, respectively, and were identified in a database of *Brassica napus* ESTs that were generated at the Saskatoon Research Centre of Agriculture and Agri-Food Canada (www.brassica.ca).

Expression and purification of recombinant HDA19

25 The open reading frame (ORF) of the *HDA19* was PCR amplified, sequence analyzed, inserted in-frame with the GST (glutathione s-transferase) into the vector pGEX-6P-2 (Amersham Pharmacia), and transformed into *E. coli* BL21-CodonPlus (DE3)-RP competent cells (Stratagene) as previously described (Gao *et al.*, 2003). The recombinant HDA19 protein was expressed and purified under non-denaturing
30 conditions as described by Gao *et al.*, 2002). The GST-HDA19 fusion protein was analyzed by western blotting with rabbit anti-GST-Pi polyclonal antibody (Chemicon) using ECL Western blotting analysis system (Amersham Pharmacia).

In vitro protein interaction assays

The entire coding region of *BnSCL1* and four fragments, *BnSCL1*¹⁻³⁵⁸, *BnSCL1*¹⁻²⁶¹, *BnSCL1*¹⁻²¹⁷, and *BnSCL1*¹⁻¹⁴⁵ encoding amino acids 1-434, 1-358, 1-261 and 1-217, respectively, were amplified by PCR and cloned into the *HindIII* and *XhoI* sites of the expression vector pET-28b (Novagen) in-frame with the His-Tag sequence. The primers used for amplification were as follows:

Forward primer for *BnSCL1*, *BnSCL1*¹⁻³⁵⁸, *BnSCL1*¹⁻²⁶¹, *BnSCL1*¹⁻²¹⁷ and *BnSCL1*¹⁻¹⁴⁵:

5'- GCAAGCTTATGGACGAACATGCCATGCGTTCCA-3' (SEQ ID NO:90)

Reverse primer for *BnSCL1*:

5'- CGCTCGAGAAAGCGCCACGCTGACGTGGC-3' (SEQ ID NO:91)

Reverse primer for *BnSCL1*¹⁻³⁵⁸:

5'- CGCTCGAGCGCGGAGATCTTCGGACGTAA-3' (SEQ ID NO:92)

Reverse primer for *BnSCL1*¹⁻²⁶¹:

5'- CGCTCGAGCCTAATCGCCTTGAAAGATAA-3' (SEQ ID NO:93)

Reverse primer for *BnSCL1*¹⁻²¹⁷:

5'- CGCTCGAGCGCCACAACCGCCGTGACTCT-3' (SEQ ID NO:94)

Reverse primer for *BnSCL1*¹⁻¹⁴⁵:

5'- CGCTCGAGCGCTCGGATCTTCTGAACAAT-3' (SEQ ID NO:95).

The TNT–Quick Coupled Transcription/Translation System (Promega) was used to produce the full-length BnSCL1 protein and the truncated mutants ΔBnSCL1¹⁻³⁵⁸, ΔBnSCL1¹⁻²⁶¹, ΔBnSCL1¹⁻²¹⁷ and ΔBnSCL1¹⁻¹⁴⁵ labeled with [³⁵S]methionine as previously described (Gao *et al.*, 2003). *In vitro* protein interaction was detected with GST pulldown affinity assays as described by Ahmad *et al.* (1999) and Gao *et al.*, (2003).

In vivo protein interaction assays

The six DNA fragments, *BnSCL1*¹⁻³⁵⁸, *BnSCL1*¹⁻²⁶¹, *BnSCL1*¹⁻²¹⁷, *BnSCL1*¹⁻¹⁴⁵, *BnSCL1*¹⁴⁶⁻³⁵⁸ and *BnSCL1*²¹⁸⁻⁴³⁸ and the ORF of *BnSCL1* encoding amino acids 1-358, 1-261, 1-217, 1-145, 146-358, 218-434 and 1-434, respectively, were PCR amplified and cloned into the *SalI* and *NotI* sites of pPC86 vector (GibcoL BRL) in-frame with the *GAL4* AD sequences to generate constructs pPC86-*BnSCL1*¹⁻³⁵⁸, pPC86-*BnSCL1*¹⁻²⁶¹, pPC86-*BnSCL1*¹⁻²¹⁷, pPC86-*BnSCL1*¹⁻¹⁴⁵, pPC86-*BnSCL1*¹⁴⁶⁻³⁵⁸, pPC86-*BnSCL1*²¹⁸⁻⁴³⁸ and pPC86-*BnSCL1*. PCR amplification was carried out using the following primers:

Forward primer for *BnSCL1*, *BnSCL1*¹⁻³⁵⁸, *BnSCL1*¹⁻²⁶¹, *BnSCL1*¹⁻²¹⁷ and *BnSCL1*¹⁻¹⁴⁵:
5'-GCGTCGACGATGGACGAACATGCCATGCGTTCCA-3' (SEQ ID NO:96)

Forward primer for *BnSCL1*¹⁴⁶⁻³⁵⁸:

5'-GCGTCGACGATTAAGGAGTTTTCCGGTATA-3' (SEQ ID NO:97)

Forward primer for *BnSCL1*²¹⁸⁻⁴³⁴:

5'-GCGTCGACGGAGGATTGCGCCGTCGAGACG-3' (SEQ ID NO:98)

Reverse primer for *BnSCL1* and *BnSCL1*²¹⁸⁻⁴³⁴:

5'-GCGCGGCCGCAAAGCGCCACGCTGACGTGGC-3' (SEQ ID NO:99)

Reverse primer for *BnSCL1*¹⁻³⁵⁸:

5'-GCGCGGCCGCGCGGAGATCTTCGGACGTAA-3' (SEQ ID NO:100)

Reverse primer for *BnSCL1*¹⁻²⁶¹:

5'-GCGCGGCCGCCCCTAATCGCCTTGAAAGATAA-3' (SEQ ID NO:101)

Reverse primer for *BnSCL1*¹⁻²¹⁷:

5'-GCGCGGCCGCGCCACAACCGCCGTGACTCT-3' (SEQ ID NO:102)

Reverse primer for *BnSCL1*¹⁻¹⁴⁵:

5'-GCGCGGCCGCGCTCGGATCTTCTGAACAAT-3' (SEQ ID NO:103)

Reverse primer for *BnSCL1*¹⁴⁶⁻³⁵⁸:

5'-GCGCGGCCGCCGCGGAGATCTTCGGACGTAA-3' (SEQ ID NO:100).

For *in vivo* protein interaction assays, the MaV203 yeast competent cells carrying the *lacZ* reporter gene were co-transfected with the construct pDBLeu-HDA19, in which the HDA19 was fused in-frame with GAL4 DB and either of the plasmids pPC86-BnSCL1, pPC86-BnSCL1¹⁻³⁵⁸, pPC86-BnSCL1¹⁻²⁶¹, pPC86-BnSCL1¹⁻²¹⁷, pPC86-BnSCL1¹⁻¹⁴⁵, pPC86-BnSCL1¹⁴⁶⁻³⁵⁸, pPC86-BnSCL1²¹⁸⁻⁴³⁸ and or the vector pPC86 alone. The expression of *lacZ* reporter gene was quantified by measuring the β -galactosidase activity using CPRG (chlorophenol red- β -D-galactopyranoside) according to the manufacturer's instructions (GibcoL BRL). Three yeast control strains A, B, and C (GibcoL BRL) that contain plasmid pairs expressing fusion proteins with none, weak and moderately strong interaction strengths, respectively, were used as controls.

15 Transactivation assay

MaV203 yeast cells expressing the *lacZ* reporter gene driven by a promoter containing GAL4 DNA binding sites (GibcoL BRL) were transformed with the pDBLeu-bnKCP1¹⁻¹⁶⁰, pDBLeu-bnKCP1¹⁻⁸⁰, pDBLeu-bnKCP1⁸¹⁻²¹⁵ and pDBLeu-bnKCP1. These vectors were constructed by ligating the PCR-amplified fragments, Δ BnSCL1¹⁻³⁵⁸, Δ BnSCL1¹⁻²⁶¹, Δ BnSCL1¹⁻²¹⁷, Δ BnSCL1¹⁻¹⁴⁵, Δ BnSCL1¹⁴⁶⁻³⁵⁸ and Δ BnSCL1²¹⁸⁻⁴³⁴ and the entire coding region of *BnSCL1*, respectively, into the *Sa*II and *Not*I sites of the vector pDBLeu (GibcoL BRL) in-frame with the GAL4 DB sequence. The oligonucleotide primers for the amplification were the same as those used for the *in vivo* protein interaction assays. The β -galactosidase activity was measured using CPRG according to the manufacturer's instructions (GibcoL BRL). In addition to the yeast strains A, B and C, the yeast strains D (GibcoL BRL) that contain plasmid pairs expressing fusion protein with strong interaction strength was used as controls.

30 Cloning and Sequence Analysis of the *BnSCL1* Gene

HDAC or HAT is recruited to specific loci by large protein complexes made up of transcription activators/co-activators and repressors/co-repressors, respectively (See reviews Kuo and Allis, 1998; Meyer, 2001). Identification of these transcription regulatory proteins that interact with HDAC or HAT is a direct approach to defining nuclear factors that recruit these chromatin remodelling regulators to their target promoters and hence affect the expression of the target genes. To isolate proteins that bind to HDAC in *B. napus*, the ORF of *Arabidopsis thaliana* HDA19 fused to the yeast Gal4 DNA binding domain was used as bait in a yeast two-hybrid screening of a *B. napus* cDNA library linked to the yeast Gal4 activation domain. A number of positive clones were obtained on the basis of the induction of three yeast reporter genes *HIS3*, *URA3* and *lacZ* followed by retransformation and sequencing analysis. One of these clones encodes a 51.2 kDa protein with pI 5.1, designated BnSCL1 (*Brassica napus* SCARECROW-like protein 1; SEQ ID NO:81). As shown in figure 20, BnSCL1 contains several domains of the SCARECROW (SCR) family of transcription factors (Laurenzio et al., 1996).

Sequence analysis revealed that BnSCL1 cDNA (2781 bp) contains two open reading frames (ORFs). The first ORF (ORF1) encodes BnSCL1, a polypeptide of 461 amino acid residues starting at 82 bp from the 5' end, and ORF2 codes for a polypeptide of 281 amino acids starting at 1687 bp from the 5' end. The linking region of the two ORFs is a short sequence of 200 bp. Database search using NCBI blast program (Altschul et al., 1997) indicated that the deduced amino acid sequence encoded by ORF2 was similar to the human polyposis coli region hypothetical protein DP1 (accession number A39658), which contains a TB2_DP1_HVA22 domain. However, the GENESCAN program (Burge and Karlin, 1997) predicts that the 2781 bps of BnSCL1 cDNA encodes one polypeptide only, i.e. the deduced amino acid sequence of ORF1.

Comparison of the deduced BnSCL1 amino acid sequence to the NCBI (<http://www.ncbi.nlm.nih.gov>) and TAIR (arabidopsis.org) databases results in a list of proteins with considerable similarity (Fig. 21). According to the NTI computer program (InforMax, Inc.), BnSCL1 shares an 89% amino acid identity with AtSCL15 (Pysh et al., 1999) or VHS5 (Silverstone et al., 1998), an *Arabidopsis* SCARECROW-like protein (accession number Z99708, At4g36710), while it is 37% identical to

AtSCR (accession number U62797). Interestingly, it also shares high similarity (66% sequence identity) with a tomato (*Lycopersicon esculentum*) protein (accession number AF273333), a member of the GRAS/VHID protein family, encoded by the Lateral suppressor gene (Ls) (Schumacher et al., 1999) (Fig. 20). Consistent with these data, phylogenetic analysis using either NTI Vector or DNA Star program classified BnSCL1, AtSCL15 and LsSCL (Ls) in the same subgroup (Fig. 21).

The *BnSCL1* copy number in *B. napus* was estimated using DNA gel blot analysis on total genomic DNA digested with restriction endonucleases and hybridized with the ORF of *BnSCL1* under high stringency conditions (Fig. 22). Digestion with *EcoRI*, *XbaI*, *HindIII*, *PstI* and *KpnI* resulted in the detection of about three bands, whereas digestion with *EcoRV* generated approximately six bands due to the existence of an internal cutting site for *EcoRV* within the *BnSCL1* gene. This result indicates that *BnSCL1* belongs to a small gene family of approximately three members in the *B. napus* genomes.

BnSCL1 is a Member of GRAS/VHID Family

The BnSCL1 gene encodes a polypeptide of 461 amino acids with several suggestive functional domains or motifs (Fig. 20). It has two MAT $\alpha 2$ -like nuclear localization signals (NLSs) (residues 169-173 and 436-440) (Raikhel, 1992). It also has a LXXLL motif (¹⁴⁸LGSL¹⁵² (SEQ ID NO:104)) that was shown to mediate interaction of transcription coactivators with nuclear receptors (Heery et al., 1997). Amino acid sequence analysis also revealed that BnSCL1 has the characteristic structure for GRAS/VHID regulatory proteins (Pysh et al., 1999), including a VHID motif that encompasses a putative NLS, two leucine heptad repeats (LHRs) that surround the conserved VHID motif, a PFYRE motif and a C-terminal SAW motif that encompasses a putative NLS (Fig. 20). The LHRI-VHID-LHRII region has been thought to function in protein-protein and DNA-protein interactions (Pysh et al., 1999).

BnSCL1 Interacts Physically with HDA19 *in vitro* and *in vivo*

To confirm the interaction of BnSCL1 protein with HDA19 that was detected in the yeast two-hybrid system, GST pulldown affinity assays were carried out using *in vitro*-translated BnSCL1 labeled with [³⁵S]Methionine. The BnSCL1 protein was tested for its binding ability to GST-HDA19 fusion protein that was expressed in *Escherichia coli* and purified under non-denaturing conditions. As shown in figure 23, BnSCL1 bound to recombinant HDA19 protein, while it did not bind to GST alone (data not shown).

To map the protein binding domain of the BnSCL1 protein, four C-terminal truncated mutants of BnSCL1 lacking either of the SWA, PFYRE, LHRII or VHID motif (Fig. 23a) were constructed. These truncated mutants were assayed for *in vitro* interaction with the recombinant HDA19 protein. As shown in Figure 23b, the mutant proteins exhibited interaction with GST-HDA19 fusion protein with the truncation from C-terminal end until the VHID region was deleted, indicating that the VHID domain is essential for BnSCL1 protein binding to HDA19.

The requirement of the VHID domain for protein-protein interaction was also demonstrated *in vivo* using the yeast two-hybrid system (Fig. 24). MaV203 yeast cells were co-transformed with plasmid pDBLeu-HDA19 and either pPC86-BnSCL1, pPC86-BnSCL1¹⁻³⁵⁸, pPC86-BnSCL1¹⁻²⁶¹, pPC86-BnSCL1¹⁻²¹⁷, pPC86-BnSCL1¹⁻¹⁴⁵, pPC86-BnSCL1¹⁴⁶⁻³⁵⁸, pPC86-BnSCL1²¹⁸⁻⁴³⁸ and or the vector pPC86 alone. Although β -galactosidase activity was reduced by at least 50% when pDBLeu-expressing cells were transformed with plasmids expressing either of the six mutants of BnSCL1 protein, as compared to the wild type BnSCL1, the transformants with plasmids expressing either pPC86-BnSCL1¹⁻¹⁴⁵ or pPC86-BnSCL1²¹⁸⁻⁴³⁸, both of which lacked VHID motif, showed a further at least 50% reduction in β -galactosidase activity as compared to the other mutants. This finding indicates that VHID domain is critical for BnSCL1 interaction with HDA19 *in vivo*.

BnSCL1 Activates Transcription of a Reporter Gene in Yeast

To further characterize the biological function of BnSCL1, its functions as a transcription activator was investigated. Transactivation experiments were performed

in yeast (Fig. 25), whereby a yeast strain carrying three reporter genes, *lacZ*, *HIS3* and *URA3*, driven by promoters fused to GAL4 DNA binding sites and independently integrated into the yeast genome were transfected with the effector plasmid pDBLeu-BnSCL1 comprising *BnSCL1* fused to the GAL4 DB under the control of the ADH promoter. Transformation with the effector plasmid resulted in increasing β -galactosidase activity similar with yeast strain D that contains plasmid pairs expressing fusion proteins with strong protein-protein interaction and approximately 20-fold relative to either vector pDBLeu alone or yeast control strain A, which contains plasmid pairs expressing fusion proteins without protein-protein interaction (Fig. 25). Reporter genes *HIS3* and *URA3* were also strongly transactivated by BnSCL1 protein (data not shown). These results indicate that BnSCL1 significantly exhibits transcription activator activity in yeast.

To map the transactivation domain of the BnSCL1 activator, a series of deletion mutants of BnSCL1 protein were generated (Fig. 25a) and used in *in vivo* transactivation assays in yeast. As shown in figure 6b, either of the deletions from C-terminal of BnSCL1 or any truncation from the N-terminal resulted in a decrease of at least 85% in β -galactosidase activity relative to the wild type BnSCL1 protein. This demonstrates that the transactivation domain of bnKCP1 may reside in both the N- and C-terminal regions.

BnSCL1 Gene is Expressed Mainly in Roots

The expression pattern of the *BnSCL1* gene was analyzed by RNA gel blot analysis and quantitative RT-PCR using total RNA extracted from various organs of *B. napus* (Fig. 26). As shown in figure 26a, there were two *bnSCL1* transcripts of 1.6 kb and 2.8 kb in the RNA blot probed with the ORF of *BnSCL1*, suggesting the existence of either two species of *BnSCL1* cDNA produced by alternative splicing in *B. napus* genome or a *BnSCL1* homologue cross-hybridizing to the probe. Both of them accumulated at highest levels in roots, whereas its expression was weak in flowers and stems, and undetectable in leaves and siliques. Results obtained using quantitative RT-PCR analysis (Fig. 26b) were consistent with those obtained with northern blotting. In addition, RT-PCR analysis revealed strong expression in seedling

shoots (Fig. 26b). This expression pattern is similar to that of *Arabidopsis* SCR gene (Laurenzio et al., 1996) and to those of most SCLs (Pysh et al., 1999). This suggests that BnSCL1 and SCR may share similar functions in the regulation of root development.

5

BnSCL1 Responds to Auxin Treatment

The plant hormone auxin plays an important role in cell division, cell elongation, cell differentiation, lateral root initiation and gravitropism (Davies, 1995; Berleth and Sachs, 2001; Liscum and Stowe-Evans, 2000). Recent studies have demonstrated that auxin distribution organizes the pattern and polarity in the root meristem (Sabatini et al., 1999). To determine whether the dominant role of SCARECROW-like proteins (SCLs) in root biology is associated with auxin, quantitative RT-PCR was used to examine the expression of BnSCL1 gene in four-leaf stage- and 10 dpg-seedlings treated with the synthetic auxin 2,4-D. As shown in Figure 27, *BnSCL1* mRNA accumulation increased by approximately 50% within 30 min of application of 1 mM 2,4-D, and then decreased rapidly to a lower level, when compared to untreated plants (Fig. 27).

Auxin levels are known to modulate the degradation rate of Aux/IAA (auxin/indole-3-acetic acid protein) family members through a proteolytic regulation mechanism (Zenser et al., 2001). To examine whether auxin levels also influences the expression pattern of *BnSCL1* gene, quantitative RT-PCR was used to analyse total RNA isolated from shoots and roots of 10 dpg seedlings treated with variable concentrations of 2,4-D ranging from 1 pM to 1 mM (Fig. 28). Expression of *BnSCL1* in shoots was rapidly downregulated by auxin even at the lowest level (1 pM) of 2,4-D, indicating that *BnSCL1* response to auxin is very sensitive (Fig. 28a). *BnSCL1* expression in roots, however, was upregulated by auxin although application of a higher concentration (100 μ M) of auxin was required to produce an effect (Fig. 28b). To determine whether response of *BnSCL1* gene to auxin was due to the exogenous application rather than the intercellular auxin synthesis, seedlings were treated for 24 h with 50 μ M of naphthylphthalamic acid (NPA), a polar auxin transport inhibitor, and the expression of *BnSCL1* in response to auxin was analysed using quantitative One-Step RT-PCR. As can be seen in Figure 28c, the *BnSCL1* mRNA accumulation

profiles were not changed both in shoots and in roots after NPA treatment followed by the application of auxin at different concentrations. These results suggest that the response of *BnSCL1* to the application of exogenous auxin was tissue-specific, or the expression of *BnSCL1* may be regulated by auxin distribution in plants.

5

Expression of SCR in apical meristems was found to be controlled by chromatin assembly factor-1 (CAF-1) (Kaya et al., 2001), and auxin gene expression mutations to be located within an *Arabidopsis* RPD3-like histone deacetylase gene, HDA6, using map-based cloning approach (Murfett et al., 2001). However, no alterations in gene expression of endogenous auxin response genes were detected in the mutants and no effect of auxin-inducible GUS expression was found after seedlings were treated with HDAC inhibitor sodium butyrate at concentration up to 1 mM for 24 h (Murfett et al., 2001). To determine whether *BnSCL1* response to auxin is modulated by HDA19, 9 dpg seedlings were treated with 2,4-D at concentrations ranging from 10^{-6} to 10^3 μ M or treated with 50 mM of sodium phosphate buffer as control after sodium butyrate treatment for 24 h at a concentration of 10 mM. Relative expression was investigated using quantitative One-Step RT-PCR to analyze RNA extracted from shoots and roots of seedlings. As shown in Figure 28, although the expression pattern of *BnSCL1* in response to auxin in shoots was different from that in roots, the inhibition of histone deacetylase led to the expression profiles of *BnSCL1* in shoots were similar to those in roots, i.e. the expression was upregulated by auxin at concentration of 1 pM and downregulated by auxin at higher concentrations. The fact that HDAC inhibition led to the alteration of *BnSCL1* expression in response to auxin suggests that the response of *BnSCL1* to auxin is modulated by histone deacetylase.

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These results suggest a molecular mechanism by which *BnSCL1* functions as a transcription factor to regulate gene expression by recruiting HDAC to the promoter regions of target genes.

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Example 6: Modulation of activity of a gene of interest using a recruitment factor

Two constructs are prepared: 1) an activator+reporter construct (Fig. 29B) carrying the *lacZ* reporter gene downstream from a Tet operator sequence (Tet-7X); and the *BnSCL1* and *VP16* genes encoding a VP16-SCL fusion protein that is able to bind the Tet operator sequence; and 2) an effector construct carrying the *HDA19* gene (Fig. 29B).

The activator+report construct is introduced and expressed in yeast cells, for example MaV203 cells as described in Example 4, to produce a reporter yeast. Activity of *lacZ* product is quantified by measuring the β -galactosidase activity using chlorophenol red- β -D-galactopyranoside (CPRG) (GibcoL BRL). In the reporter yeast, expression of the activator+reporter construct results in the expression of the VP16-SCL fusion protein that binds to the Tet operator sequence, thereby activating expression of the *LacZ* reporter gene due to VP16.

The reporter yeast expressing the activator+reporter construct is treated with tetracycline. Expression of *lacZ* reporter gene is quantified by measuring the β -galactosidase activity using chlorophenol red- β -D-galactopyranoside (CPRG). The expression of the activator+reporter construct in the presence of tetracycline in yeast cells produces a baseline level of LacZ activity.

The effector construct is then introduced into the reporter yeast so that the activator+reporter and the effector constructs are both expressed, and the activity of the *LacZ* product determined as indicated above. Results demonstrate that LacZ activity is reduced in the yeast expressing both the activator+reporter and the effector constructs, when compared to LacZ activity determined in the reporter yeast expressing only the activator+reporter construct, and approximates the level of activity of LacZ activity produced by the reporter yeast when treated with tetracycline.

This result indicate that the expression of a gene of interest (in this case *LacZ*) may be reduced by targeting a recruitment factor, for example SCL1, to the nucleotide sequence encoding the gene of interest, and permitting the recruitment factor to bind an HDAC.

A similar set of assays is carried out comprising three constructs: 1) a reporter construct carrying the *lacZ* reporter gene, 2) an activator construct carrying the *BnSCL1* and *VP16* genes, and 3) an effector construct carrying the *HDA19* gene (see Fig. 29A). The constructs are expressed in yeast cells, for example MaV203 cells as described above in Example 4, in the following combinations:

- reporter construct alone,
- reporter and activator constructs,
- reporter, activator and effector constructs.

The expression of *lacZ* reporter gene is quantified by measuring the β -galactosidase activity using chlorophenol red- β -D-galactopyranoside (CPRG) (GibcoL BRL).

The expression of the reporter construct alone in yeast cells produces a baseline level of β -galactosidase activity. Expression of both the reporter and activator constructs yields an elevated level of β -galactosidase activity, when compared with the activity observed in the presence of the reporter construct alone, while the reporter, activator and effector constructs together results in approximately background levels of β -galactosidase activity.

All citations are herein incorporated by reference.

The present invention has been described with regard to preferred embodiments. However, it will be obvious to persons skilled in the art that a number of variations and modifications can be made without departing from the scope of the invention as described herein.

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